Association of the *OPRM1* variant rs1799971 (A118G) with non-specific liability to substance dependence in a collaborative *de novo* meta-analysis of European-ancestry cohorts

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ABSTRACT

The mu1 opioid receptor gene, *OPRM1*, has long been a high-priority candidate for human genetic studies of addiction. Because of its potential functional significance, the non-synonymous variant rs1799971 (A118G, Asn40Asp) in *OPRM1* has been extensively studied, yet its role in addiction has remained unclear, with conflicting association findings. To resolve the question of what effect, if any, rs1799971 has on substance dependence risk, we conducted collaborative meta-analyses of 25 datasets with over 28,000 European-ancestry subjects. We investigated non-specific risk for "general" substance dependence, comparing cases dependent on any substance to controls who were non-dependent on all assessed substances. We also examined five specific substance dependence diagnoses: DSM-IV alcohol, opioid, cannabis, and cocaine dependence, and nicotine dependence defined by the proxy of heavy/light smoking (cigarettes-per-day > 20 versus ≤ 10). The G allele showed a modest protective effect on general substance dependence (OR = 0.90, 95% C.I. [0.83-0.97], p-value = 0.0095, N = 16,908). We observed similar effects for each individual substance, although these were not statistically significant, likely because of reduced sample sizes. We conclude that rs1799971 contributes to mechanisms of addiction liability that are shared across different addictive substances. This project highlights the benefits of examining addictive behaviors collectively and the power of collaborative data sharing and meta-analyses.

KEY WORDS: Addiction; substance dependence; *OPRM1*; opioid receptor; single nucleotide polymorphism (SNP); genetic association

1. INTRODUCTION

The mu opioid receptors are part of a family of G protein-coupled receptors that are expressed in the brain and bind endogenous and exogenous opioids. The mu1 opioid receptor gene (*OPRM1*) has been one of the most studied genes in psychoactive substance research. It is a receptor for opioid analgesic agents and is involved in reward and analgesic pathways (Kreek and Koob 1998). The non-synonymous single nucleotide polymorphism (SNP) rs1799971 (A118G) in exon 1 of *OPRM1* causes an asparagine to aspartic acid substitution at the fortieth amino acid residue (Asn40Asp). The G (Asp) allele is the minor allele across multiple human populations, with frequencies ranging from 4% in African-American samples to ~16% in European-ancestry samples to over 40% in some Asian samples (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1799971). Multiple studies have examined the functional effects of this amino acid change on expression levels and receptor properties such as binding affinity and signaling (Befort et al. 2001; Beyer et al. 2004; Bond et al. 1998; Deb et al. 2010; Mague and Blendy 2010; Mague et al. 2009; Ray et al. 2012; Wang et al. 2014; Wang et al. 2012; Zhang et al. 2005).

Because of its potential functional significance, many human genetic studies of substance dependence have targeted rs1799971. However, the role, if any, of rs1799971 in substance dependence remains unclear (Crist and Berrettini 2013; Levran et al. 2012; Mague and Blendy 2010). In studies of opioid dependence, results have been mixed, with the minor (G) allele reported to have no effect in some studies (Crowley et al. 2003; Levran et al. 2008; Nelson et al. 2014; Nikolov et al. 2011) and to decrease risk in others (Bond et al. 1998; Tan et al. 2003). Similarly, analyses of alcohol dependence have reported increased risk (Bart et al. 2005; Kim et al. 2004), no effect (Bergen et al. 1997; Rouvinen-Lagerstrom et al. 2013; Sander et al. 1998;

Xuei et al. 2007), and decreased risk (Schinka et al. 2002; Town et al. 1999) for this allele. Analyses of rs1799971 with other addictive substances also show no consensus (Clarke et al. 2013; Crist and Berrettini 2013; Franke et al. 2001; Gelernter et al. 1999; Hardin et al. 2009; Munafo et al. 2013).

Literature-based meta-analyses have evaluated the association of rs1799971 with substance dependence (Arias et al. 2006), opioid dependence (Coller et al. 2009; Glatt et al. 2007; Haerian and Haerian 2013), and alcohol dependence (Chen et al. 2012a). Three of these meta-analyses reported no association (Arias et al. 2006; Coller et al. 2009; Glatt et al. 2007), while among Asian samples the G allele was reported to increase risk for alcohol (Chen et al. 2012a) and opioid dependence (Haerian and Haerian 2013). Although these meta-analyses attained large samples by combining published information, they were subject to heterogeneity from multiple sources, including differing phenotypes, ascertainment schemes, and statistical analysis models across the meta-analyzed publications.

To clarify the effect of rs1799971 on substance dependence risk, we conducted collaborative meta-analyses based on new analyses of multiple datasets. Our data-driven approach moves beyond the limitations of literature-based meta-analyses by (1) defining consistent phenotypes across studies, (2) performing new, uniform analyses across datasets as in our previous meta-analyses (Chen et al. 2012b; Hartz et al. 2012; Saccone et al. 2010), and (3) inviting investigators to contribute analyses from established studies with relevant phenotype and genotype data, irrespective of prior publication on rs1799971.

2. METHODS

2.1. Samples and Study design

Twenty-five datasets contributed a starting sample of 28,689 study participants of European ancestry. Invitations to participate were sent to all studies in the NIDA Genetics Consortium, which NIDA formed to facilitate collaboration among investigators in addiction genetics, as documented by the NIDA Center for Genetic Studies (https://nidagenetics.org/studies). We extended invitations to additional studies suggested by consortium members as likely to have relevant data, and to collaborators on a previous meta-analysis of smoking quantity and lung disease (Saccone et al. 2010). NIDA further advertised the opportunity to participate in this meta-analysis project with a web announcement at http://www.drugabuse.gov/researchers/research-resources/genetics-research-resources/collaborative-opportunities-genetics-research. Dataset inclusion criteria were: (1) rs1799971 must have been genotyped, and (2) at least one of these five phenotypes must have been assessed: DSM-IV defined alcohol, cannabis, cocaine, or opioid dependence, or categorized cigarettes per day (CPD) (0-10, 11-20, 21-30, and 31+ CPD).

Study participants with a history of abstinence from alcohol (never drank) were excluded prior to all analyses, so that included participants satisfied a minimum exposure to alcohol. For the main analyses, we filtered out study participants if they had no known substance dependence and were also under the age of 25. Thus, we included non-dependent (control) participants only if they were old enough to have passed through the period of highest risk, so as to reduce phenotypic misclassification. For each dataset, Table 1 gives demographic characteristics, the allele frequency of rs1799971, and key publications. Supplementary text S1 provides additional details for each dataset, including study recruitment, genotyping methods, and data quality control.

2.2. Phenotypes

We analyzed six primary dichotomous phenotypes: a "general" substance dependence diagnosis (lifetime dependence on any of five substances: alcohol, nicotine, cannabis, cocaine, and opioids), plus the corresponding five individual substance-specific lifetime dependence diagnoses. General substance dependence controls were required to be non-dependent on all substances assessed in that dataset; not all studies assessed all five substances. For each substance, individuals who did not meet dependence criteria were classified as non-dependent; abuse criteria were not considered. These phenotypes allowed us to examine the general (non-specific) liability to substance dependence and compare non-specific and substance-specific associations.

DSM-IV criteria were used to define dependent cases for alcohol, cannabis, cocaine, and opioids. For nicotine dependence, we defined the proxy of heavy smoking cases (CPD > 20) and light smoking controls (CPD ≤ 10) for current and former smokers, based on CPD when they were smoking; if multiple measurements were available the maximum value was used. Heavy versus light CPD is more commonly measured than nicotine dependence and has been an informative proxy for nicotine dependence in large meta-analyses (Chen et al. 2012b; Hartz et al. 2012; Saccone et al. 2010); smokers meeting this threshold strongly overlap with nicotine dependent smokers. Because CPD does not account for dependence items such as withdrawal (Lessov et al. 2004), secondary analyses examined the effect of redefining general dependence using standard definitions of nicotine dependence (Fagerström Test for Nicotine Dependence (Heatherton et al. 1991) and DSM-IV), in the subset of studies for which these were available.

In addition to filtering out subjects who did not meet minimum exposure to alcohol, we also defined analysis variables for exposure to each of the other four substances. For cannabis, cocaine, and opioids, the exposure threshold was "at least one lifetime use." For nicotine, we

used “at least 100 cigarettes smoked lifetime,” a commonly used threshold to define smoking exposure in epidemiological studies.

Table 2 shows dataset-specific counts for cases, controls, and exposed controls. Individuals dependent on multiple substances are counted and analyzed in the corresponding multiple categories.

2.3. SNP for analysis

We required rs1799971 to be genotyped in each dataset. For analyses, we coded rs1799971 as the number of copies of the G (minor) allele.

2.4. Statistical analyses and meta-analysis

We conducted six correlated discovery tests corresponding to the six primary phenotypes: the general substance dependence diagnosis and the five specific substance dependence diagnoses. To limit the number of tests, we focused on testing for a main effect of rs1799971 on these outcomes. All discovery analyses filtered out study participants under the age of 25 with no known substance dependence to ensure that controls had passed the typical age of dependence onset; cases are dependent and thus have had sufficient exposure regardless of age. Additional interpretive tests examined the robustness and consistency of discovery test results, and included analyses without age filtering for comparison.

To ensure uniform analyses across datasets, the coordinating site at Washington University developed analysis scripts in SAS® and R. Scripts were distributed to collaborating sites, which then analyzed their datasets locally. Results were returned to the coordinating site for meta-analyses. We used standard inverse-variance-weighted meta-analysis as implemented in the

rmeta package in R (Lumley ; R Development Core Team 2012). Additionally, to be included in the meta-analysis of a given model, each dataset was required to have at least five cases and five controls available. This requirement was intended to reduce noise when some subgroups became very small after phenotypic filtering. All samples included for general dependence in fact met a higher threshold of at least 20 cases and 20 controls. We report fixed effect estimates together with Cochran's Q and I^2 to evaluate heterogeneity for each meta-analyzed model. No significant heterogeneity was observed among the studies analyzed (p-value for $Q > 0.05$, Table S1 and Tables 3 and 4). Correspondingly, Q values were close to the respective degrees of freedom (number of studies) and I^2 values were small with no values greater than 26% (Supplementary Table S1).

2.5. General substance dependence analyses

Logistic regression was used to estimate the effect of rs1799971 on general substance dependence with covariates for sex and age. Of the 25 available datasets, 20 had at least five cases (dependent at least one of the five substances) and five controls (no known substance dependence diagnoses and exposed to alcohol) for analysis of general substance dependence.

Our interpretive tests examined the robustness of the general substance dependence results and compared them to substance-specific effects. Specifically, to assess the influence of each individual dataset, each of the 20 contributing datasets was, in turn, left out of the meta-analysis. In this leave-one-out test, observing consistency of summary odds ratios would suggest that it is unlikely that the overall meta-analysis result is primarily due to a single study. Also, we meta-analyzed only studies that had assessed all five substances to examine consistency of results; the general dependence controls in these studies were assessed for all five substances and thus more

homogenous. Finally, to compare the effect of rs1799971 on general substance dependence liability with its effect on the constituent substance-specific diagnoses, we tested for association using individuals dependent on each specific substance as cases compared to the same controls used in the general dependence analysis (non-dependent on all assessed substances).

2.6. Specific substance dependence analyses

To test the association of rs1799971 with each specific dependence diagnoses while accounting for the remaining diagnoses, our primary analysis used ordinal logistic regression with additively coded rs1799971 as the dependent variable and the five dependence diagnoses, four exposures, sex, and age as explanatory variables. This model simultaneously estimates association of rs1799971 with each substance while accounting for co-morbidity (Grucza et al. 2008). This analysis used only the datasets that had all five substance dependence diagnoses and all four exposure variables because the model required that there be no missing variables.

To interpret and examine the robustness of these results, we evaluated traditional logistic regression models on the same datasets, also accounting for co-morbidity: each specific substance dependence was tested as the outcome, with log-additively coded rs1799971, age, sex, and the remaining specific substance dependence diagnoses as explanatory variables. Here, cases were dependent on a given substance, and controls were exposed but not dependent on that substance regardless of diagnoses for the remaining four substances. Additionally, to test equivalence of regression coefficients from ordinal regression analyses of individual substances, we conducted a two sample t-test assuming unequal variance.

To examine whether substance-specific results remained consistent with a larger number of datasets, we used all datasets that had assessed each substance for additional interpretive tests,

with the dependence diagnosis as outcome and additively coded rs1799971, sex, and age as explanatory variables.

2.7. Multiple test correction

To estimate the effective number of independent tests corresponding to the six correlated discovery tests, we used matSpD [http://gump.qimr.edu.au/general/daleN/matSpD/], which accounts for correlations among phenotypes (Cheverud 2001; Li and Ji 2005; Nyholt 2004). Using Pearson correlations among the five dependence diagnoses from the studies with all five phenotypes assessed (see Table S3), plus one additional test for general substance dependence, we obtained a conservative estimate of 5.1218 independent tests, corresponding to a Bonferroni-corrected p-value threshold of $\alpha' = 9.76 \times 10^{-3}$ for statistical significance.

3. RESULTS

3.1. The G (Asp) allele of rs1799971 shows a modest protective effect on general substance dependence

We observed a significant association between rs1799971 and general substance dependence (Figure 1). Based on 9064 cases and 7844 age-filtered controls from 20 datasets, the G allele showed a modest protective effect (OR = 0.90, 95% C.I. [0.83-0.97], p-value = 9.52×10^{-3}, N = 16,908); 15 of the 20 studies showed a protective direction of the G allele. Heterogeneity variance was not statistically significant (Q=20.13, p-value = 0.39). A secondary analysis that did not require controls to be over 25 years old yielded a similar odds ratio (OR = 0.90, 95% C.I. (0.84-0.98), N = 17,918), but was not statistically significant after multiple correction in this larger sample (p-value = 1.06×10^{-2}), consistent with our hypothesis that it is important for controls to be past the typical age of risk.

Leave-one-out test of robustness yielded odds ratio estimates ranging from 0.88 to 0.92, with none of the 20 iterations showing significant heterogeneity. This tight range of ORs centered on the overall odds ratio indicates that our finding was not driven by a single dataset. Only a few of the individual iterations showed significant association (e.g. 4 of 20 when using $\alpha' = 9.76 \times 10^{-3}$ as the significance threshold), likely due to the reduced sample size.

To reduce potential heterogeneity among the general dependence controls, we meta-analyzed the 10 datasets that had all five substance-specific dependence diagnoses and at least 5 cases and 5 controls. For these 10 datasets (3947 cases and 2348 controls), the summary odds ratio was 0.87 (p-value = 0.01), very similar to the discovery result based on 20 studies.

Additionally, to aid interpretation, we compared the cases for each specific substance to the general dependence controls. We found that the G allele of rs1799971 was consistently protective (odds ratio of 0.83 to 0.93) across all five substances (Table 3), consistent with the interpretation that this allele is a non-substance-specific protective factor.

To further confirm robustness, we examined the effect of redefining general dependence using alternative definitions for nicotine dependence, namely the Fagerström Test for Nicotine Dependence (FTND) (case ≥ 4, control ≤ 1; 13 studies, N = 8,481) or DSM-IV nicotine dependence (14 studies, N = 11,711), in place of our CPD-based heavy/light phenotype (20 studies, N = 16,908). Analyses of these smaller samples gave similar protective odds ratios for general dependence, though results were not statistically significant: OR = 0.91, 95% C.I. (0.81-1.02) for FTND and OR = 0.94, 95% C.I. (0.85-1.03) for DSM-IV nicotine dependence.

3.2. For each substance-specific dependence, the G allele of rs1799971 is similarly protective but non-significant

In our primary test of rs1799971 genotype as the dependent variable on the 9 datasets that had assessed all five substance dependence diagnoses and exposures, we obtained odds ratios that ranged from 0.89 (nicotine dependence) to 0.92 (cocaine dependence). The odds ratio for each specific substance showed the same protective direction as that for general substance dependence, though none was statistically significant in these smaller samples (Table 4). Also, odds ratios for specific substances did not differ significantly from each other (Table S2), suggesting consistency across substances.

We also examined traditional logistic regression in these 9 datasets. Each substance dependence diagnosis was examined as the outcome (cases dependent on that substance and controls required to be non-dependent but exposed to that substance), with rs1799971 as the predictor and the remaining diagnoses as covariates. Results were similar to those from our ordinal logistic model (Table 4, bottom half). Finally, analyzing all available datasets for these same case/control outcomes (cases dependent on each specific substance, controls non-dependent and exposed to that substance) also showed protective, but non-significant, odds ratios consistent with those seen in the datasets that assessed all dependence diagnoses and exposures (Supplementary Figures S1-S5).

4. DISCUSSION

This project, the first collaborative genetic meta-analysis to investigate specific and general liability for these substance dependence diagnoses, has demonstrated that the G allele of rs1799971 has a modest protective effect on general substance dependence liability (OR = 0.90, 95% C.I. (0.83-0.97), p-value = 9.52×10^{-3}) in samples of European ancestry. This is the first meta-analysis to show that this non-synonymous variant, which has been heavily studied for

functional effects, is significantly associated in European ancestry samples with liability to substance dependence. The small but significant effect size of rs1799971 suggests that variability in previous association reports may be due in part to sampling variation. This collaborative meta-analysis benefited from the opportunity to define uniform phenotypes across studies, perform coordinated, *de novo* analyses to test our hypotheses, and include existing datasets that have not yet focused on the question of rs1799971 and addiction.

The protective effect of this allele on substance dependence liability appears to be non-specific: it is not driven primarily by dependence on any particular substance. For each substance-specific subset of cases compared to the general dependence controls, we observed a protective effect of similar size to that observed for general dependence. Additional substance-specific analyses similarly showed consistent protective effects of the G allele. These substance-specific odds ratios were not statistically significant, but this may have been largely due to reduced sample size and power.

These findings indicate that rs1799971 in *OPRM1* may contribute to mechanisms of addiction liability that are shared across different addictive substances, consistent with the high genetic correlation between the traits, high co-morbidity, and with prior studies showing that both substance-specific and non-specific genetic effects on addiction liability can be expected (Bierut et al. 1998; Kendler et al. 2007; Merikangas et al. 1998; Swan et al. 1997; Tsuang et al. 1998; Vanyukov et al. 2012; Vanyukov et al. 2003). Rs1799971 is now one of the few examples of a genetic factor that demonstrates a similar, general effect across multiple substances, albeit of modest magnitude. In this sense, our study is similar to a genome-wide association study of multiple psychiatric disorders that identified variants having a common, cross-disorder genetic effect on five major psychiatric diseases (Cross-Disorder Group of the Psychiatric Genomics

Consortium 2013). Both studies underscore the value of investigating the genetics of general liability underlying related diseases. Genetic studies of addiction would therefore benefit from including measures pertaining to multiple substances that can then be analyzed collectively. Indeed, a very recent genome-wide study of general substance dependence liability using four of the five substances studied here (alcohol, cannabis, cocaine, opioids) reported novel associations (Wetherill et al. 2015), further supporting the potential benefits.

Our results are compatible with negative results from prior genome-wide meta-analyses of cigarettes-per-day (Liu et al. 2010; The Tobacco and Genetics Consortium 2010; Thorgeirsson et al. 2010). Our hypothesis-driven analyses of a single SNP translate to a study-wide required significance threshold of 9.76×10^{-3}. This led to statistically significant evidence for a modest effect (OR=0.90) of rs1799971 on general substance dependence liability, in N=16,908 subjects (Table 3). The three genome-wide smoking consortia tested *OPRM1* only in each consortium separately (N=38,000, N=31,000, and N=16,000 smokers with cigarettes-per-day); estimated power to have detected the nicotine-specific odds ratio of 0.93 (Table 3) in at least one of the three consortia with genome-wide significance (alpha=5×10^{-8}) is only 4%. Power details are in Supplementary Text S2. Hence it is not surprising that these smoking consortia did not report an *OPRM1* effect.

This study contributes valuable information to connect functional findings to the clinically important outcome of addiction in humans. Several neurobiological, functional, and physiological changes have been demonstrated for the rs1799971 (A118G) amino acid change and a corresponding mutation in a similar region of the receptor in mice (A112G) (Drakenberg et al. 2006; Huang et al. 2012; Mague and Blendy 2010; Palmer and de Wit 2012; Ray et al. 2012; Wang et al. 2014). *In vitro* studies of the G allele have reported increased binding to β-

endorphin (Bond et al. 1998), altered downstream signaling (Deb et al. 2010), and decreased mu opioid receptor expression (Zhang et al. 2005). In human brain imaging, the G allele is associated with striatal dopamine response to alcohol (Ramchandani et al. 2011) and increased mu opioid receptor binding potential (Ray et al. 2011). In mouse knock-in models (A112G), the G/G knock-in has shown reduced receptor protein levels overall and reduced reinforcing value of morphine in female mice (Mague et al. 2009), reduced G-protein signaling (Wang et al. 2014), and increased peak dopamine response to alcohol challenge (Ramchandani et al. 2011); changes are often brain-region specific.

It is important to note that some functional and neurobiological findings have been interpreted as indicating that the G allele of rs1799971 should increase risk for addiction, for example due to its association with greater alcohol-induced reinforcement and reward (Ramchandani et al. 2011; Ray and Hutchison 2004; Ray and Hutchison 2007; Ray et al. 2010). Our data-driven evidence of a modest *protective* effect of this allele on substance dependence liability is thus surprising and all the more important to integrate with functional findings to understand downstream contributions to human substance dependence. A protective effect of the G allele on addiction may be consistent with either increased or decreased reward/reinforcement, for example due to varying roles of positive versus negative reinforcement at different stages in the transition from use to dependence. Modeling these connections remains an open area to be worked out by neurobiological theories of addiction (Ray et al. 2012).

This project demonstrates the value of collaborative data sharing and meta-analysis, as the modest odds ratio of rs1799971 would be challenging to detect and consistently replicate in modestly sized candidate gene studies (Hall et al. 2013; Hart et al. 2013). Also important was our approach of defining consistent phenotypes across all datasets. In particular, careful

definition of controls can help to detect associations (Nelson et al. 2013; Schinka et al. 2002). In our case, requiring controls to be at least 25 years of age led to stronger association results even with the reduced the number of controls.

This study has limitations. First, as in any meta-analysis, sample heterogeneity could not be completely avoided. Studies had diverse ascertainment schemes, with some designed to recruit dependent cases for one particular substance. Some studies recruited from the general population while others recruited potentially more extreme cases from treatment centers. Hence, over- and under-representation of phenotypes were present in contributing datasets, and the severity of dependence, degree of co-morbid dependence, and prevalence of substance exposure varied. Reduced proportions of exposed controls would reduce effective sample size and power for a study. But overall, uniform phenotype definitions were an important design feature to ameliorate effects of heterogeneity. Although some bias may have occurred, it seems unlikely to have been systematic in either direction. Similarly, it seems unlikely that systematic bias would have occurred due to differences between studies that contributed to this meta-analysis and those that declined to participate.

Second, this project interrogated only the non-synonymous variant rs1799971. As with any statistical association, our finding may reflect a proxy association for which the true functional variant(s) remain to be recognized. Other *OPRM1* variants have been associated with addiction and merit consideration for future study (Clarke et al. 2013; Hancock et al. 2015; Zhang et al. 2006a). Analyses of multiple SNPs and haplotypes will also be of future interest: recent evidence indicates an important role in heroin addiction for the haplotype structure of *OPRM1*, with the A allele of rs1799971 showing association only in the presence of the C allele of rs3778150 (Hancock et al. 2015). Importantly, (Hancock et al. 2015) also found that the G allele of

rs1799971 is protective (A allele confers risk) on that background, agreeing with the direction of effect observed in our meta-analysis of general substance dependence.

Third, further phenotypic refinement is possible. We did not consider substance abuse criteria, nor did we use the newer diagnostic system, DSM-5 (American Psychiatric Association 2013). Our threshold for exposure was a single use for all substances except nicotine; therefore, the genetic effect of rs1799971 detected by our analyses may involve a combination of effects on development of regular/repeated use and effects on dependence. We focused on dichotomous diagnoses for each substance. For nicotine, we examined heavy/light smoking as the most widely available nicotine trait in our datasets. Consistency of results was confirmed using DSM-IV and Fagerström Test of Nicotine Dependence criteria when available. Because we focused on dichotomous diagnoses that could then be combined into the general substance dependence diagnosis, we did not examine quantitative or categorical cigarettes-per-day.

Fourth, we focused on main effects of rs1799971 to limit multiple testing. Thus, we did not examine gene-environment interactions (e.g., sex-specific effects) or gene-gene interactions. We did adjust statistically for sex, which showed no evidence for a main effect on general substance dependence ($p = 0.57$). Interactions likely have roles in a complex trait such as addiction, and could attenuate the genetic main effect when not accounted for (e.g. when the effect occurs only in a specific stratum). Thus, it is possible that the modest main effect that we detected could translate to a stronger effect if particular genetic or environmental backgrounds are considered. Future work could examine interactions nominated in the literature (Mague et al. 2009; Miranda et al. 2013; Ray et al. 2006).

Finally, a model that explicitly partitions the association between a general factor for any substance dependence and substance-specific components was not fitted to these data. Although such a model would allow a more refined distinction between general and specific associations (Medland and Neale 2010; Neale et al. 2006), we chose not to apply this because of the complexities of running and integrating such analyses across sites.

In closing, this data-driven, collaborative meta-analysis has demonstrated a modest protective effect of the G allele of rs1799971 on general liability to substance dependence. This work highlights the benefits of jointly studying related disorders: larger samples and insight into factors involved in underlying shared liability. An important strength of our approach is that the analyses of our datasets were designed and conducted in collaboration with the originating investigators. Thus, we benefited from collaborators' deep knowledge of their own data and our combined expertise on addiction. This effort underscores the value of collaboratively sharing data and expertise to accelerate discoveries.

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Conflict of Interest Disclosures: Dr. Bierut is listed as an inventor on Issued U.S. Patent 8,080,371, "Markers for Addiction" covering the used of certain SNPs in determining the diagnosis, prognosis, and treatment of addiction, and served as a consultant for Pfizer in 2008. Dr. NL Saccone is the spouse of Dr. SF Saccone, who is also listed as an inventor on the above patent. Dr. Cinciripini served on the scientific advisory board of Pfizer, conducted educational talks sponsored by Pfizer on smoking cessation (2006-2008), and has received grant support from Pfizer. Dr. Degenhardt has no relevant disclosures for this specific project; however, for general pharmaceutical company disclosures, Dr. Degenhardt has received untied educational grants from Reckitt Benckiser to conduct post-marketing surveillance of the diversion and injection of opioid substitution therapy medications in Australia. Although these activities are unrelated to the current study, Dr. Kranzler has been a consultant or advisory board member for Alkermes, Lilly, Lundbeck, Otsuka and Pfizer; he is also a member of the American Society of

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Informed Consent. The procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 and 2008. All participants provided informed consent.

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Figure 1
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Figure 1. Forest plot of general substance dependence and rs1799971 across studies that had at least 5 cases and 5 controls. Summary odds ratio, 95% Confidence Interval, and p-values are based on fixed effect meta-analysis. *indicates the subset of 10 studies that had all five specific substance dependence diagnoses, examined in secondary analyses to confirm consistency of results. Estimated heterogeneity variance was Q = 20.13 with a p-value of 0.387 among all 20 studies and Q = 6.49 with a p-value of 0.69 among the subset of 10 studies.

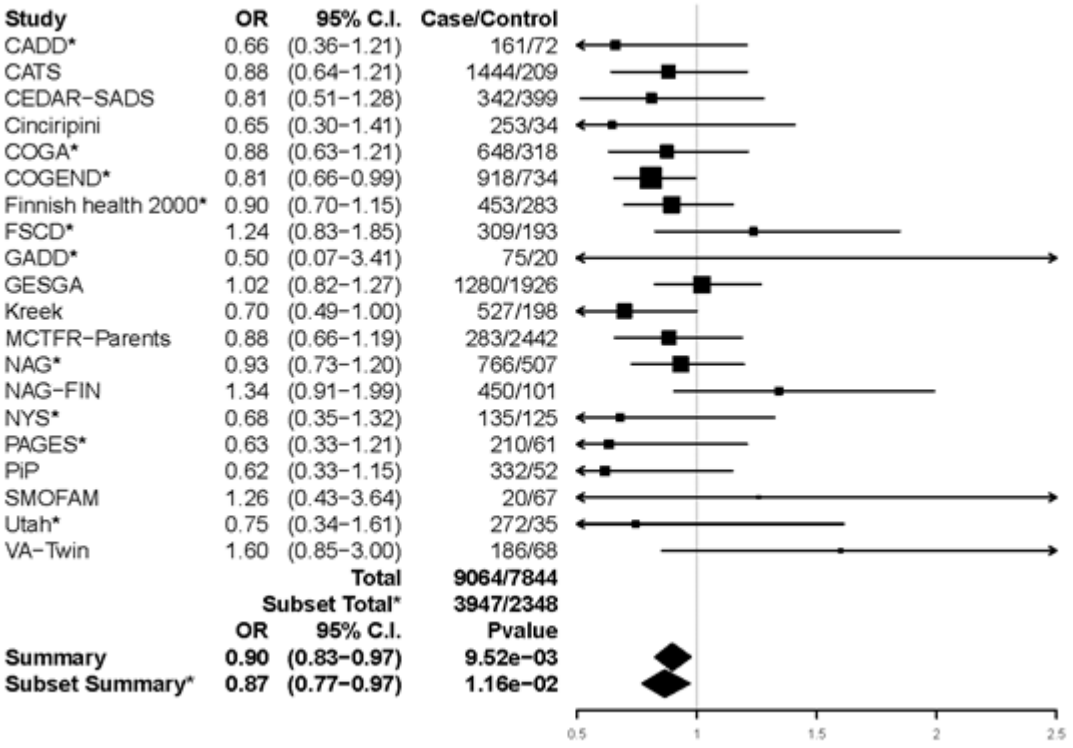

Table 1: Study descriptions: sample sizes, G (minor) allele frequencies (overall, in general dependence cases, in general dependence controls), proportions of male participants, and age distribution (minimum, first quartile, median, third quartile, and maximum) of each study.

Study	Total N	G-allele-freq	Case G-allele-freq	Control G-allele-freq	proport-male	age min	age_q1	age_med	age_q3	age_max
BG [a]	3999	0.14	0.13	0.17	0.83	18	25	28	30	58
CADD [b]	409	0.13	0.12	0.18	0.56	12	21	28	47	72
CATS [c]	1748	0.12	0.12	0.13	0.57	18	29	36	43	65
CEDAR-SADS [d]	747	0.12	0.12	0.12	0.35	14	18	37	42	65
Cinciripini [e]	627	0.11	0.09	0.15	0.48	18	33	42	50	74
COGA [f]	1024	0.12	0.11	0.13	0.46	18	36	43	51	77
COGEND [g]	2024	0.13	0.13	0.15	0.61	25	32	37	41	65
Finnish Health 2000 [h]	1025	0.21	0.20	0.22	0.8	30	37	46	54	87
FSCD [i]	558	0.13	0.13	0.10	0.5	18	25	34	40	54
FT12 [j]	617	0.22			0.48	20	22	22	23	27
GADD [k]	281	0.12	0.13	0.15	0.57	12	15	16	19	61
GESGA [l]	3501	0.12	0.11	0.11	0.65	18	39	47	56	84
Kreek [m]	750	0.11	0.10	0.13	0.59	17	31	43	52	82
MCTFR-Parents [n]	3842	0.11	0.10	0.11	0.46	30	43	46	50	72
NAG-AUS [o]	1281	0.12	0.12	0.12	0.59	18	36	43	50	82
NAG-FIN [p]	879	0.21			0.7	42	52	55	58	77
NYS [q]	552	0.1	0.07	0.10	0.49	35	37	39	41	44
OYSUP [r]	357	0.13			0.5	20	21	21	21	23
PAGES [s]	409	0.11			0.68	17	27	37	45	68
PiP [t]	809	0.11	0.11	0.15	0.48	18	35	43	53	79
ROMA [u]	732	0.21	0.16	0.38	0.83	18	25	28	32	53
SMOFAM [v]	166	0.12	0.18	0.12	0.63	26	27	29	30	62
Utah [w]	463	0.13	0.12	0.14	0.59	25	54	61	67	86
VA-Twin [x]	672	0.12	0.15	0.10	0.32	21	30	38	46	58
Yale-Penn [y]	1217	0.12			0.41	16	30	39	46	72
Total	**28689**	**(0.10-0.22)**			**(0.32-0.83)**	**(12-42)**	**(15-54)**	**(16-61)**	**(19-67)**	**(23-87)**

References: [a] (Nikolov et al. 2011); [b] (Stallings et al. 2005; Stallings et al. 2003); [c] (Nelson et al. 2014; Nelson et al. 2013); [d] (Maher et al. 2011); [e] (Carter et al. 2008; Cinciripini et al. 2006; Cinciripini et al. 2005; Lam et al. 2012; Minnix et al. 2011; Robinson et al. 2007; Robinson et al. 2011); [f] (Edenberg 2002; Edenberg et al. 2010); [g] (Saccone et al. 2009a; Saccone et al. 2009b); [h] (Aromaa and Koskinen 2004; Rouvinen-Lagerstrom et al. 2013); [i] (Bierut et al. 2010; Bierut et al. 2008); [j] (Broms et al. 2012; Kaprio et al. 2002); [k] (Kamens et al. 2013); [l] (Frank et al. 2012; Treutlein et al. 2009); [m] (Levran et al. 2008); [n] (Iacono et al. 1999; Keyes et al. 2009; McGue et al. 2007; Miller et al. 2012); [o] (Loukola et al. 2008; Saccone et al. 2007); [p] (Loukola et al. 2008); [q] (Elliott et al. 1985; Elliott et al. 1989; Hoft et al. 2009); [r] (Andrews et al. 2003); [s] (Van den Oord et al. 2006); [t] (David et al. 2011); [u] (Nikolov et al. 2011); [v] (Hops et al. 2000); [w] (Weiss et al. 2008); [x] (Chen et al. 2009; Kendler et al. 2007; Zhang et al. 2006b); [y] (Gelernter et al. 2014; Gelernter et al. 2013a; Gelernter et al. 2013b)

Table 2. Numbers of cases, total controls, and controls with exposure to each substance. These numbers were based on a filtered sample that removed participants not exposed to alcohol, and participants who are less than 25 years of age and have with no dependence to any assessed substances. NA indicates that the substance was not assessed in the study.

	Alcohol		**Cigarettes Per Day (CPD)**			**Cannabis**			**Cocaine**			**Opioid**			**General Substance Dependence**	
Datasets	Cases	Total Controls	Heavy Smokers	Total Controls	Exposed Controls	Cases	Total Controls	Exposed Controls	Cases	Total Controls	Exposed Controls	Cases	Total Controls	Exposed Controls	Cases	Controls
BG	277	1278	1449	4	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	948	3
CADD	93	264	54	109	57	72	285	218	35	306	141	6	351	106	161	72
CATS	651	1032	NA	NA	NA	857	831	765	416	1272	875	1259	429	102	1444	209
CEDAR-SADS	179	562	NA	NA	NA	255	486	338	86	655	263	114	627	418	342	399
Cinciripini	NA	NA	253	34	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	253	34
COGA	612	410	242	483	140	196	823	475	224	795	204	86	933	193	648	318
COGEND	463	1529	584	935	923	192	1808	1543	132	1871	560	34	1970	191	918	734
Finnish Health 2000	417	512	89	463	180	3	1011	0	0	1014	0	2	1012	0	453	283
FSCD	280	230	124	276	58	170	340	213	237	273	55	81	429	100	309	193
FT12	93	470	198	15	15	NA	NA	NA	NA	NA	NA	NA	NA	NA	251	0
GADD	43	110	11	57	NA	45	108	78	9	144	29	NA	153	35	75	20
GESGA	1333	1933	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	1280	1926
Kreek	42	684	NA	NA	NA	NA	NA	NA	82	644	41	528	198	NA	527	198
MCTFR-Parents	226	2504	NA	NA	NA	93	2637	1637	37	2695	452	12	2722	125	283	2442
NAG-AUS	359	972	737	594	102	79	1249	694	5	1322	74	17	1310	137	766	507
NAG-FIN	221	513	354	115	115	NA	NA	NA	NA	NA	NA	NA	NA	NA	450	101
NYS	65	466	71	135	63	25	508	368	26	507	157	1	532	86	135	125
OYSUP	All participants were under 25 years of age															
PAGES	64	335	132	83	40	77	320	160	7	391	67	8	390	30	210	61
PiP	NA	NA	347	54	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	332	52
ROMA	18	240	209	7	6	NA	NA	NA	NA	NA	NA	NA	NA	NA	177	4
SMOFAM	NA	NA	20	67	34	NA	NA	NA	NA	NA	NA	NA	NA	NA	20	67
Utah	112	283	235	60	60	36	359	88	16	379	34	9	386	9	272	35
VA-Twin	NA	NA	193	70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	186	68
Yale-Penn	799	208	1094	113	NA	397	566	500	911	259	167	703	478	214	1208	3
Total	6347	14535	6396	3674	1797	2497	11331	7077	2223	12527	3119	2860	11920	1746	11648	7834

Table 3. Summary of the effect of rs1799971 on general substance dependence.

Model	Cases	Controls	Cochrane's Q	Q-Pvalue	Odds Ratio	L95%-U95%	OR-Pvalue
Gen-Dep = age sex rs1799971	9064	7844	20.13	0.387	0.90	0.83-0.97	0.952
Alcohol = age sex rs1799971	5086	7623	12.08	0.672	0.92	0.83-1.01	0.696
Nicotine = age sex rs1799971	3358	2670	16.84	0.265	0.93	0.83-1.05	0.244
Cannabis = age sex rs1799971	2077	5115	7.63	0.746	0.83	0.71-0.98	0.279
Cocaine = age sex rs1799971	1307	5313	7.68	0.809	0.87	0.73-1.04	0.132
Opioid = age sex rs1799971	2139	5168	7.87	0.641	0.84	0.70-1.00	0.557

Model column shows what outcome phenotype was tested for each model. Gen-Dep denotes general substance dependence. Each substance denotes the subsets of general substance dependence that were tested in interpretative phase of the analysis. All effects shows are fixed effect estimates. Controls were filtered for age and exposure to alcohol.

Table 4. Summary of the effect of rs1799971 on specific substance dependence diagnoses in 9 studies that assessed all five substance dependence diagnoses and exposures.

Ordinal Logistic Regression Results							
Substance	**Cases**	**Controls**	**Cochrane's Q**	**Q-Pvalue**	**Odds Ratio**	**L95%-U95%**	**OR-Pvalue**
Alcohol	2031	3361	8.90	0.351	0.90	0.76-1.06	0.218
Nicotine	2718	2674	7.78	0.455	0.89	0.74-1.07	0.216
Cannabis	839	4553	10.76	0.216	0.91	0.73-1.14	0.420
Cocaine	992	4085	0.86	0.990	0.92	0.69-1.24	0.593
Opioid	607	4274	3.12	0.682	0.91	0.65-1.27	0.577
Traditional Logistic Regression Results (Dependence as outcome variable)							
Alcohol	2051	3430	10.66	0.222	0.88	0.76-1.02	0.974
Nicotine	2066	1412	8.69	0.276	0.91	0.76-1.08	0.267
Cannabis	861	3036	9.08	0.336	0.90	0.74-1.09	0.283
Cocaine	1011	899	0.85	0.997	0.91	0.70-1.19	0.492
Opioid	600	577	2.31	0.679	0.91	0.67-1.24	0.547

Substance column shows the tested outcome phenotype. All effects shows are fixed effect estimates. In the traditional logistic regression results, controls were required to be exposed each tested substance, in addition to meeting the previously applied filters for age and exposure to alcohol.

Supplementary Text S1: Dataset Descriptions.

GENETIC EPIDEMIOLOGY OF OPIOID DEPENDENCE IN BULGARIA (BG / GEODBG) [1]

GEODBG is a collaboration of sites in the United States and in Bulgaria. Subjects were recruited from major Bulgarian cities, primarily Sofia and Plovdiv. Participants had at least 1 year history of daily heroin use.

The MAGIC was used to obtain DSM-IV lifetime diagnoses for abuse/dependence for alcohol, heroin and other illicit drugs. Nicotine consumption measures were also collected.

For this meta-analysis, GEODBG contributed rs1799971 genotypes from 2,098 unrelated ethnic Bulgarians heroin dependent and 1,901 ethnic Bulgarian population controls (BG dataset); and from 424 heroin dependent Bulgarian Romas and 308 Roma population controls (ROMA dataset). The study obtained informed consent from participants and approval from the appropriate institutional review boards at Washington University and Sofia Medical University.

DNA was extracted from venous blood samples from each heroin dependent subject, and from 10mm punches (PKU Guthrie cards) for controls, using chemagic Magnetic Separation Module I (chemagen AG) according to manufacturer's protocol. A Biomek FX robot was used for DNA aliquoting in 384 well plates and TaqMan assays for genotyping the 118A>G polymorphism on a 7900HT Fast Real-Time PCR System (both from Applied Biosystems). SDS v2.2.2 analysis software tool was used for base-calling and visualization of the genotype data. For quality control purposes, four samples from each plate were re-genotyped on another plates (no errors were detected). The call rate for the five case and the four control 384 plates was over 95%.

FAMILY, TWIN, AND ADOPTION STUDIES OF THE COLORADO CENTER ON ANTISOCIAL DRUG DEPENDENCE (CADD) [2-3]

The Colorado Center on Antisocial Drug Dependence (CADD) sample encompasses over 5000 individuals that have been studied in a longitudinal design to gain a better understanding of the genetic mechanisms of adolescent drug abuse. The full CADD sample consists of both clinical and community populations [2-3]. The subjects used in the current analyses were from the Colorado Adolescent Substance Abuse (ASA) family study. Clinical probands were recruited from three treatment facilities in the Denver metropolitan area. The probands were 13-19 years of age at time of assessment and were drawn from individuals with consecutive admissions to the treatment facilities between February 1993 and June 2001. Community probands were matched to the clinical probands based on age, gender, ethnicity and zip code. At the time of assessment all individuals living in the same household as the proband were interviewed. All study participants were given cognitive, psychiatric, and socio-demographic assessments that included both structured diagnostic interviews as well as self-reported questionnaires. Since the samples were drawn from a study of related individuals SAS (proc surveyselect) was used to randomly select one individual per family for inclusion in the current analysis. Substance use patterns were assessed with the Composite International Diagnostic Interview—Substance Abuse Module (CIDI-SAM), a structured, face-to-face diagnostic assessment designed to be administered by trained, lay interviewers [4]. This assessment procedure has been shown to be valid for adolescent subjects [5]. From the CIDI-SAM the alcohol, cocaine, nicotine, opioid and cannabis

DSM-IV dependence were examined. Additionally the number of cigarettes smoked per day at the time of maximum smoking was also analyzed. All research was approved by the institutional review board of the University of Colorado. Genomic DNA was isolated from buccal cells using a modification of published procedures [6-8]. Taqman assays for allelic discrimination (Applied Biosystems, Foster City, CA) were used to determine SNP genotypes. QC included the removal of individuals with fewer than 90% genotypes across all SNPs tested in these individuals (assumed poor quality DNA sample). Genotypes were called by two independent individuals and removed in case of a disagreement.

COMORBIDITY AND TRAUMA STUDY (CATS) [9-10]

The Comorbidity and Trauma Study (CATS), a collaboration of investigators at Washington University School of Medicine, Queensland Institute of Medical Research, and National Drug and Alcohol Research Centre of the University of New South Wales, is a case-control genetic association study of heroin dependence. Heroin dependent cases were recruited from opioid replacement therapy clinics in the greater Sydney region. Controls with little or no lifetime history of recreational opioid use were recruited from economically-disadvantaged neighborhoods in geographic proximity to the clinics.

Psychiatric diagnostic interviews modified from the Semi-Structured Assessment for the Genetics of Alcoholism were completed in-person and included sections on illicit drug and alcohol dependence from which exposure information and DSM-IV lifetime diagnoses of opioid, cannabis, sedative, stimulant, cocaine, and alcohol abuse and dependence were obtained. CATS contributed a sample of 1259 heroin dependent cases and 429 controls for this meta-analysis. All participants, unrelated individuals of European ancestry who were successfully genotyped for rs1799971, had provided informed consent. Institutional review board approval was obtained from the three primary institutions and the area health service ethics committees governing participating clinics.

DNA was extracted from whole blood samples. Genotyping was performed on an Illumina BeadStation using GoldenGate technology. Details of data cleaning have been reported previously [9-10]. In brief, SNPs were excluded due to genotyping failure, call rate less than 95%, minor allele frequency less than 2%, and Hardy-Weinberg deviations. Data from samples were excluded due to phenotypic-genotypic gender mismatch, duplication due to participation in the project multiple times, and cryptic relatedness with identity by descent greater than 0.5. Principal components analyses conducted using the smartpca program in the Eigensoft 3.0 package [11] facilitated removal of individuals from the sample who were not of European ancestry.

CENTER FOR EDUCATION AND DRUG ABUSE RESEARCH (CEDAR) – SUBSTANCE ABUSE AND THE DOPAMINE SYSTEM STUDY (SADS) [12]

CEDAR and SADS are United States projects. Participants were recruited from the Pittsburgh metropolitan area through newspaper advertisements, social service agencies, substance abuse treatment programs and various other media.

For this project, analyses were conducted on a combined sample of from two studies with distinct but related ascertainment schemes, from the same Greater Pittsburgh population, joined in the *Substance Use Disorder Liability: Candidate System Genes* study (R01 DA019157). CEDAR (P50 DA005605) is a longitudinal family/high-risk study of substance use disorder (SUD) [12]. Both CEDAR and SADS studies focus on general (non-drug-specific) liability to SUD [13-14]. Parents from a sample of nuclear families, ascertained in CEDAR through the father who did or did not have a DSM-III-R SUD (DSM-IV was introduced after this study started) related to illicit drugs (an illegal substance or nonmedical use of a prescribed psychoactive drug), provided a source for male and female cases and controls. An expanded version of the Structured Clinical Interview for DSM-III-R-outpatient version (SCID-OP) [15] was administered by experienced research associates to obtain psychiatric diagnoses for adults in CEDAR. All diagnoses have been revised using DSM-IV criteria, and the SADS participants were diagnosed accordingly. To evaluate smoking behavior, Fagerström Test for Nicotine Dependence (FTND) was administered. Control subjects had no substance (including alcohol) use disorder, or Axis I or II psychiatric disorder. Probands in the SADS study (R01 DA011922) were males 12-18 years of age having a DSM-IV diagnosis of substance dependence related to use of illicit drugs. In both CEDAR and SADS subsamples, probands having a psychiatric disorder other than SUD qualified for the study unless they had a lifetime history of psychosis or any other condition where valid reporting was uncertain. The vocabulary subscale of WISC-III (subjects below age 16) or WAIS-III (age 16 and older) was administered prior to implementation of the protocol and was required to be in the normal range (>70). Since psychiatric comorbidity is common among substance abusers, cases were not excluded for any Axis I or Axis II disorders. The CEDAR and SADS subjects were self-identified European-Americans from the same Greater Pittsburgh geographic area, and the genomic inflation factor based on all genotyped SNPs, evaluating the excess false-positive rate, was satisfactory at .98[12].

For this meta-analysis, CEDAR contributed a sample of 757 unrelated European-Americans who were genotyped for rs1799971. The study obtained informed consent from participants and approval from the University of Pittsburgh Institutional Review Board.

DNA was extracted from whole blood. Genotyping of the DNA samples was carried out using the Illumina BeadArray platform in a custom 1536 SNP oligonucleotide pool assay covering 106 genes representing major neurobiological systems (candidate system genes). The procedures involved and quality control are described previously [12] and in the online Appendix to that article. Individuals missing > 20% of genotype data and then SNPs with 5% missing rates were dropped from the dataset.

CINCIRIPINI OPRM1 [16-22]

1.1 CASSI

Participants for CASSI were recruited from the University of Texas MD Anderson Cancer Center through community-based telephone screening to determine eligibility for the study. The participants were treatment-seeking adult smokers who were enrolled in a study to evaluate the efficacy of a handheld computer-assisted gradual smoking reduction treatment.

The presence of Nicotine Dependence disorder was assessed using self-reported DSM-IV criteria. Additionally, nicotine dependence severity was estimated using the Fagerström Test for Nicotine Dependence (FTND) and self-reported cigarettes smoked per day (CPD).

For this meta-analysis, CASSI contributed an unrelated sample of 112 African-American and 408 European-Americans who were genotyped for rs1799971. The study obtained informed consent from participants and approval from the MD Anderson institutional review board.

1.2 Genomic DNA was extracted from buccal cells by using the QIAmp DNA kit (cat. # 51185; QIAGEN Sciences,Valencia, CA). Single nucleotide polymorphism genotyping of the DA and nAChR SNPs was performed using the 5' nuclease assay to discriminate between the two alleles representing the different genotypes. The assay reagents for SNP genotyping consisted of a mix of PCR primers and probes. OPRM1 (Probe 1: FAM-CTTAGATGGCGACCTGT; Probe 2: VIC-CTTAGATGGCAACCTGT; Primer 1: CGGTTCCTGGGTCAACTTGTC; Primer 2: GTTCGGACCGCATGGGT) polymorphism was genotyped in assay-by-design. Each assay enables scoring of both alleles in a single well within a 384-well plate. All assays are optimized to work with genomic DNA and TaqMan Universal Master Mix. Forty cycles of PCR were performed and analyzed using an ABI Prism 7900HT Sequence Detection System from Applied Biosystems.

1.3 SCOPE

Participants for SCOPE were recruited from the University of Texas MD Anderson Cancer Center through community-based telephone screening to determine eligibility for the study. The participants were treatment-seeking adult smokers who were enrolled in a study to assess the efficacy of venlafaxine as a smoking cessation pharmacotherapy [18].

The presence of Nicotine Dependence disorder was assessed using self-reported DSM-IV criteria. Additionally, nicotine dependence severity was estimated using the Fagerström Test for Nicotine Dependence (FTND) and self-reported cigarettes smoked per day (CPD).

For this meta-analysis, SCOPE contributed an unrelated sample of 6 African-American and 116 European-Americans who were genotyped for rs1799971. The study obtained informed consent from participants and approval from the MD Anderson institutional review board.

1.4 Genomic DNA was extracted from buccal cells by using the QIAmp DNA kit (cat. # 51185; QIAGEN Sciences,Valencia, CA). Single nucleotide polymorphism genotyping of the DA and nAChR SNPs was performed using the 5' nuclease assay to discriminate between the two alleles representing the different genotypes. The assay reagents for SNP genotyping consisted of a mix of PCR primers and probes. OPRM1 (Probe 1: FAM-CTTAGATGGCGACCTGT; Probe 2: VIC-CTTAGATGGCAACCTGT; Primer 1: CGGTTCCTGGGTCAACTTGTC; Primer 2: GTTCGGACCGCATGGGT) polymorphism was genotyped in assay-by-design. Each assay enables scoring of both alleles in a single well within a 384-well plate. All assays are optimized to work with genomic DNA and TaqMan Universal Master Mix. Forty cycles of PCR were performed and analyzed using an ABI Prism 7900HT Sequence Detection System from Applied Biosystems.

1.5 PEERS EMA

Participants for PEERS EMA were recruited from the University of Texas MD Anderson Cancer Center through community-based telephone screening to determine eligibility for the study. The participants were non-treatment-seeking adult smokers who were enrolled in a study to assess real-time cigarette craving and mood before and after smoking [16].

The presence of Nicotine Dependence disorder was assessed using self-reported DSM-IV criteria. Additionally, nicotine dependence severity was estimated using the Fagerström Test for Nicotine Dependence (FTND) and self-reported cigarettes smoked per day (CPD).

For this meta-analysis, PEERS EMA contributed an unrelated sample of 42 African-American and 26 European-Americans who were genotyped for rs1799971. The study obtained informed consent from participants and approval from the MD Anderson institutional review board.

1.6 Genomic DNA was extracted from buccal cells by using the QIAmp DNA kit (cat. # 51185; QIAGEN Sciences,Valencia, CA). Single nucleotide polymorphism genotyping of the DA and nAChR SNPs was performed using the 5' nuclease assay to discriminate between the two alleles representing the different genotypes. The assay reagents for SNP genotyping consisted of a mix of PCR primers and probes. OPRM1 (Probe 1: FAM-CTTAGATGGCGACCTGT; Probe 2: VIC-CTTAGATGGCAACCTGT; Primer 1: CGGTTCCTGGGTCAACTTGTC; Primer 2: GTTCGGACCGCATGGGT) polymorphism was genotyped in assay-by-design. Each assay enables scoring of both alleles in a single well within a 384-well plate. All assays are optimized to work with genomic DNA and TaqMan Universal Master Mix. Forty cycles of PCR were performed and analyzed using an ABI Prism 7900HT Sequence Detection System from Applied Biosystems.

1.7 PEERS NS

Participants for PEERS NS were recruited from the University of Texas MD Anderson Cancer Center through community-based telephone screening to determine eligibility for the study. The

participants were non-treatment-seeking adult smokers who were enrolled in a study to assess psychophysiological response to nicotine nasal spray [17, 20-21].

The presence of Nicotine Dependence disorder was assessed using self-reported DSM-IV criteria. Additionally, nicotine dependence severity was estimated using the Fagerström Test for Nicotine Dependence (FTND) and self-reported cigarettes smoked per day (CPD).

For this meta-analysis, PEERS NS contributed an unrelated sample of 40 African-American and 32 European-Americans who were genotyped for rs1799971. The study obtained informed consent from participants and approval from the MD Anderson institutional review board.

1.8 Genomic DNA was extracted from buccal cells by using the QIAmp DNA kit (cat. # 51185; QIAGEN Sciences,Valencia, CA). Single nucleotide polymorphism genotyping of the DA and nAChR SNPs was performed using the 5' nuclease assay to discriminate between the two alleles representing the different genotypes. The assay reagents for SNP genotyping consisted of a mix of PCR primers and probes. OPRM1 (Probe 1: FAM-CTTAGATGGCGACCTGT; Probe 2: VIC-CTTAGATGGCAACCTGT; Primer 1: CGGTTCCTGGGTCAACTTGTC; Primer 2: GTTCGGACCGCATGGGT) polymorphism was genotyped in assay-by-design. Each assay enables scoring of both alleles in a single well within a 384-well plate. All assays are optimized to work with genomic DNA and TaqMan Universal Master Mix. Forty cycles of PCR were performed and analyzed using an ABI Prism 7900HT Sequence Detection System from Applied Biosystems.

1.9 PEERS WS

Participants for PEERS WS were recruited from the University of Texas MD Anderson Cancer Center through community-based telephone screening to determine eligibility for the study. The participants were treatment-seeking adult smokers who were enrolled in a behavioral smoking cessation therapy trial to evaluate the relationship between nicotine withdrawal and post-cessation affect [19, 22].

The presence of Nicotine Dependence disorder was assessed using self-reported DSM-IV criteria. Additionally, nicotine dependence severity was estimated using the Fagerström Test for Nicotine Dependence (FTND) and self-reported cigarettes smoked per day (CPD).

For this meta-analysis, PEERS WS contributed an unrelated sample of 23 African-American and 45 European-Americans who were genotyped for rs1799971. The study obtained informed consent from participants and approval from the MD Anderson institutional review board.

Genomic DNA was extracted from buccal cells by using the QIAmp DNA kit (cat. # 51185; QIAGEN Sciences,Valencia, CA). Single nucleotide polymorphism genotyping of the DA and nAChR SNPs was performed using the 5' nuclease assay to discriminate between the two alleles representing the different genotypes. The assay reagents for SNP genotyping consisted of a mix of PCR primers and probes. OPRM1 (Probe 1: FAM- CTTAGATGGCGACCTGT; Probe 2: VIC-CTTAGATGGCAACCTGT; Primer 1: CGGTTCCTGGGTCAACTTGTC; Primer 2: GTTCGGACCGCATGGGT) polymorphism was genotyped in assay-by-design. Each assay

enables scoring of both alleles in a single well within a 384-well plate. All assays are optimized to work with genomic DNA and TaqMan Universal Master Mix. Forty cycles of PCR were performed and analyzed using an ABI Prism 7900HT Sequence Detection System from Applied Biosystems.

COLLABORATIVE STUDY ON THE GENETICS OF ALCOHOLISM (COGA) [23-24]

The Collaborative Study on the Genetics of Alcoholism (COGA) is a large, ongoing family-based study that includes subjects recruited by seven sites around the US: Indiana University, State University of New York Health Science Center Brooklyn, University of Connecticut, University of Iowa, University of California San Diego, Washington University St Louis, and Howard University [23]. Probands were recruited from alcoholism treatment facilities, and after obtaining informed consent, additional family members were also contacted. Comparison families were recruited from the same communities by a variety of methods. The institutional review boards of all participating institutions approved the study. Assessment involved a detailed personal interview developed for this project, the Semi-Structured Assessment for the Genetics of Alcoholism [25] (SSAGA), which gathers detailed information on alcoholism related symptoms along with other drugs and psychiatric symptoms, from which lifetime diagnoses based on DSM-IV were derived.

A case-control sample of unrelated subjects was drawn from the COGA dataset[24]. Cases were drawn from the families ascertained through alcohol dependent probands; all cases met DSM-IV criteria for alcohol dependence. Controls all had consumed alcohol but did not meet a diagnosis of alcohol use, abuse, dependence or harmful use by any of 4 diagnostic systems (Feighner, DSM-IIIR, DSM-IV, ICD10), nor a diagnosis of abuse or dependence on cannabis, opioids, cocaine, sedatives, or stimulants and did not share a known common ancestor with a case[24]. DNA sources included blood and lymphoblastoid cell lines. Subjects were genotyped at the Center for Inherited Disease Research (CIDR) using Illumina HumanHap1M Bead-Chips; data cleaning has been described [24].

COLLABORATIVE GENETIC STUDY OF NICOTINE DEPENDENCE (COGEND) [26-27]

The Collaborative Genetic Study of Nicotine Dependence (COGEND) is a United States multi-site project. Subjects were recruited from St. Louis, Detroit, and Minneapolis through community-based telephone screening to determine eligibility for the study.

Semi-Structured Assessment of Nicotine Dependence (SSAND) was used to obtain phenotypic variables. Diagnoses and Statistical Manual of Mental Disorder IV (DSM-IV) lifetime diagnoses for alcohol, nicotine, cannabis, cocaine, and opiate were assessed using SSAND. In addition to DSM-IV diagnoses, nicotine dependences from Fagerström Test for Nicotine Dependence (FTND) and Cigarettes Per Day (CPD) measures were assessed as well. Lifetime exposure was also reported in SSAND for all five substances

For this meta-analysis, COGEND contributed a sample of 1877 unrelated European-Americans who were genotyped for rs1799971. The study obtained informed consent from participants and approval from the appropriate institutional review boards.

DNA was derived from whole blood maintained by the Rutgers University Cell and DNA Repository following stringent quality control and assurance procedures (www.rucdr.org). Genotyping of the DNA samples was carried out using Perlegen, Illumina GoldenGate, and Sequenom MassArray iPLEX technology. Cleaning procedures have been detailed [27-28]. Briefly, DNA samples with call rates < 90% were dropped; SNPs were required to pass a call rate threshold of 98%; self-reported race was verified using EIGENSTRAT [29].

FINNISH HEALTH 2000 [30-31]

Finnish Health 2000 Study [32] is a nationwide health interview and examination survey carried out in Finland in 2000-2001. A representative sample of 8028 adults, aged 30 and over, living in the mainland of Finland was produced by using two-stage stratified cluster sampling design (by the 5 university hospital regions with16 health care districts selected per region, each region containing about 1 million inhabitants i.e. 1/5 of the population). The ethics committees of the National Public Health Institute and the Helsinki and Uusimaa Hospital District accepted the ethical approval of the survey, and all participants (N=7415) provided a written informed consent.

The lifetime diagnoses for alcohol, cannabis, opiate, and cocaine dependencies based on Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV), were assessed using the computerized version of the Composite International Diagnostic Interview (M-CIDI). Overall, 5955 individuals took part to the M-CIDI diagnostic mental health interview, filled in a self-report of the seasonal changes in mood and behavior and gave venous blood samples for DNA extraction. From these individuals, 522 cases met the diagnostic criteria for alcohol dependence (422) or alcohol abuse (100). In addition, 517 healthy (DSM-IV) individuals matched by age and gender were selected as the controls. There were 101 women in both groups. In addition to DSM-IV diagnoses, as part of the survey participants answered to the questions "Have you smoked at least 100 times during your life time (cigarettes, cigars or pipe tobacco)?" and "How much on average do you smoke daily or smoked before you gave up smoking daily (factory-made cigarettes, self-rolled cigarettes, pipe tobacco, cigars)?". The latter question was recoded to the two-level cigarettes-per-day (CPD) variable.

For this meta-analysis, Finnish Health 2000 survey contributed rs1799971 genotypes for 1025 samples. Genomic DNA was isolated from whole blood using standard procedures. Genotyping was carried out using TaqMan® technology and Applied Biosystems 7300 Real Time PCR System. 2% of the samples were re-genotyped to confirm the precision of genotyping. For the analysis of alcohol dependence, the subjects with alcohol abuse diagnosis were excluded.

FAMILY STUDY OF COCAINE DEPENDENCE (FSCD) [33-34]

For FSCD study, cocaine-dependent cases were recruited systematically from chemical dependency treatment units in the greater St Louis metropolitan area. Community-based control subjects were identified through the Missouri Family Registry and matched by age, race, gender and residential zip code. Controls were biologically unrelated individuals from the same communities who consumed alcohol, but had no life-time history of dependence on any substance.

The study participants were assessed using psychiatric, lifetime diagnostic interview based on the Semi-Structured Assessment for the Genetics of Alcoholism. Informed consent was obtained from all participants by trained research assistants.

As part of GENEVA, DNA samples were genotyped on the Illumina Human 1M-Duo beadchip by the Center for Inherited Disease Research (CIDR) at Johns Hopkins University. The Illumina 1M–Duo array has a total of 1 072 820 probes, of which 23 812 are 'intensity-only', leaving 1 049 008 probes as SNP assays. These SNP assays demonstrate excellent data quality—95% of SNPs have a missing call rate <1.4% and the median of the missing call rate is 0.05%. A thorough data cleaning procedure was applied to ensure the highest possible data quality, including the use of HapMap controls, detection of gender and chromosomal anomalies, hidden relatedness, population structure, missing call rates, batch effects, Mendelian error detection, duplication error detection and Hardy–Weinberg equilibrium. Of the 1 049 008 SNPs, 948 658 SNPs passed data cleaning procedures. Further details are provided in the comprehensive data cleaning report posted on the GENEVA website (http://www.genevastudy.org/docs/GENEVA_Alcohol_QC_report_8Oct2008.pdf).

FINNTWIN12 STUDY (FT12) [35-36]

FT12 is a longitudinal birth cohort study of Finnish twins, born 1983-1987. Baseline questionnaire assessments of ca. 2,600 twin pairs were made in the year before the twins reached age 12 [35]. In a second, intensive study phase, 1852 twins who were mostly selected randomly but also included families considered to be at risk for alcohol problems were interviewed at age 14.This sample was followed-up as young adults, including a structured psychiatric interview, clinical examinations, neuropsychological testing and blood draw for genetic analyses.

The FT12 study provided information on nicotine, alcohol, cannabis, cocaine and opiate dependence, based on the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) [25, 37]. With the exception of nicotine, DSM-IV lifetime diagnoses for substance dependence were assessed using SSAGA. Nicotine dependences from Fagerström Test for Nicotine Dependence (FTND) and Cigarettes Per Day (CPD) measures were assessed. Lifetime exposure for all five substances was also reported in SSAGA. All data-collection procedures were approved by the Ethics committee of the Hospital District of Helsinki and Uusimaa, and the IRB of Indiana University, Bloomington, IN, including the use of appropriate and approved informed-consent procedures.

In the FT12 study, a total of 872 Finnish young adult twins were genotyped for rs1799971. For this meta-analysis, one twin from pairs in which both co-twins were genotyped was randomly selected for the analyses. This resulted in a sample of 617 unrelated twins (52.0% females) whose mean age at assessment was 22.4 years (sd=0.7 yrs, range: 19.9–26.5 yrs).

DNA was extracted from blood and saliva samples using standard procedures. Genotyping and quality control was performed at the Welcome Trust Sanger Institute (Hinxton, UK) on the Human670-QuadCustom Illumina BeadChip (Illumina, Inc., San Diego, CA, USA), as previously described [36].

THE GENETICS OF ANTISOCIAL DRUG DEPENDENCE (GADD) [38]

538 unrelated European American and 102 unrelated African American subjects were drawn from the Genetics of Antisocial Drug Dependence (GADD) [39] sample encompassing over 4,000 subjects. Unrelated subjects were drawn from the whole sample by randomly selecting one member per family using Statistical Analysis System (SAS) 9.3 software (SAS Institute Inc., Cary, NC). The GADD sample consists of clinical probands, aged 14-19 at first assessment, their siblings, and one or both biological parents. Probands in Denver, CO, and San Diego, CA, were identified from treatment programs, involvement with the criminal justice system, or special schools who had at least one lifetime substance dependence symptom (other than nicotine dependence) and at least one symptom of conduct disorder. The GADD is a longitudinal study in the 2nd wave of data collection; wave 1 was collected between 2001 and 2006, and wave 2, collection of which began in 2009, is currently ongoing. Only data from the completed wave 1 was used in the current study. All study participants were given cognitive, psychiatric, and socio-demographic assessments that included both structured diagnostic interviews as well as self-reported questionnaires. The University of California and the University of Colorado IRBs approved all subject recruitment, assessment, and DNA collection procedures.

Substance use patterns were assessed with the Composite International Diagnostic Interview—Substance Abuse Module (CIDI-SAM), a structured, face-to-face diagnostic assessment designed to be administered by trained, lay interviewers [4]. This assessment procedure has been shown to be valid for adolescent subjects [5]. The following DSM-IV substance dependence phenotypes were assessed: nicotine dependence, alcohol dependence, opioid dependence, cocaine dependence, and cannabis dependence. The interview includes substance use patterns, from which the phenotype of cigarettes per day was derived.

DNA was obtained with consent through either buccal cells or blood. SNP genotyping on 3,072 subjects was performed with TaqMan©® assays for allelic discrimination according to manufacturer's instructions (Applied Biosystems, Foster City, California). Polymerase Chain Reaction (PCR) reactions were performed with the Biomek® 3000 Laboratory Automation Workstation (Beckman Coulter Inc, Brea, California) and the Dual 384-Well GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, California). To analyze the amplified plates the 7900 Real-Time PCR System (Applied Biosystems, Foster City, California) was used. QC performed on the genotyped sample excluded individuals with fewer than 90% genotypes across all SNPs ever genotyped in this sample (N = 170; assumed poor quality DNA sample). Two independent individuals called all SNP genotypes; if genotype calls did not agree they were excluded.

GERMAN STUDY OF THE GENETICS OF ADDICTION (ALCOHOLISM) (GESGA) [40-41]

The German Study of the Genetics of Addiction (Alcoholism) (GESGA) is a German multi-center project. All patients fulfilled DSM-IV criteria for alcohol dependence and were recruited through consecutive admissions to the psychiatry and addiction medicine departments of German psychiatric hospitals participating in the German addiction research network [41].

Control individuals were drawn from several population based epidemiologic / community based German samples [40-41].

For this meta-analysis, GESGA contributed a sample of 3501 unrelated German individuals who were genotyped for rs1799971. The study obtained written informed consent from participants and approval from the appropriate institutional review boards.

DNA was derived from whole blood according to standard procedures. Genotyping of the DNA samples was carried out using Illumina HumanHap 550, Human610quad and Human660w quad bead chips. Cleaning procedures have been detailed [40-41]. Briefly, DNA samples with call rates < 98% were dropped; SNPs were required to pass a call rate threshold of 98%; self-reported ancestry was verified using EIGENSTRAT [29].

THE LABORATORY OF THE BIOLOGY OF ADDICTIVE DISEASES (Kreek) [42]

The "Kreek" sample includes unrelated former severe heroin addicts (cases) and normal volunteers (controls). Cases were treated at a methadone maintenance treatment program (MMTP) at the time of recruitment and had one or more years of daily multiple uses of heroin. Subjects were recruited at either the Rockefeller University Hospital or MMTPs (e.g. Manhattan Campus of VA NY Harbor Health Care System, Weill Medical College of Cornell University), and the Adelson Clinics for Drug Abuse Treatment and Research, in Las Vegas. Exclusion criteria from Control category: a) At least one instance of drinking to intoxication, or any illicit drug use in the previous 30 days; b) A past history of alcohol drinking to intoxication, or illicit drug use, more than twice a week, for more than 6 consecutive months; and c) Cannabis use for more than 12 days in the prior 30 days or past use for more than twice a week for more than 4 years (e.g. [43]). The following instruments were used: Addiction Severity Index (ASI) [44], KMSK [45], DSM-IV and a family history questionnaire of three generations.

For this meta-analysis, KREEK contributed a sample of 750 unrelated European-Americans (528 heroin dependents and 222 controls) who were genotyped for rs1799971. The study obtained informed consent from participants and approval from the appropriate institutional review board.

DNA was derived from whole blood following stringent quality control and assurance procedures. Genotyping of the DNA samples was carried out using Illumina GoldenGate [46], Taqman® and/or Sanger sequencing. Cleaning procedures have been detailed [42]. Self-reported race was verified using a family history questionnaire and STRUCTURE analysis of 178 AIMs [47].

THE MINNESOTA CENTER FOR TWIN AND FAMILY RESEARCH (MCTFR) [48-51]

The Minnesota Center for Twin and Family Research (MCTFR) is a Minneapolis-based project. Participants from twin, adoptive, and non-adoptive families were recruited from the state of Minnesota. Twins were recruited using Minnesota Department of Health birth certificate records. Mirroring the population from which the twins were sampled, approximately 95% of the twins and their families are of European ancestry. Adoptive families were ascertained through records of infant placements made by the three largest private adoption agencies in Minnesota. Non-adoptive (biological) non-twin families were located from birth certificates with families sampled randomly to obtain families with children matching the age and gender distribution of the adoptive children. While the majority of the parents in this non-twin-family sample are White, just under 40% of the siblings are East Asian. Study eligibility was established through

phone contact and a biographical questionnaire, and adolescents with mental or physical limitations that would hinder their ability to complete assessments (e.g., mental retardation, autism, blindness, etc.), who did not live with at least one biological parent if not adopted or at least one adoptive parent if adopted, or who lived more than a day's drive from the University of Minnesota's Minneapolis campus, were not recruited. No other exclusion criteria, including race or ethnic origin, were applied. Additional description of the MCTFR samples and procedures is given in [48-50]. Participants gave informed consent to participate in the MCTFR assessments, or in the case of minor children assent along with parental consent. MCTFR protocols, including those used in the genotyping analysis, have been approved by the University of Minnesota's Institutional Review Board.

For this meta-analysis, the MCTFR contributed a sample of 3846 European-Americans selected from among the parents in the MCTFR studies who had been genotyped for rs1799971. Participants in the meta-analysis were not genetically related to one another but may have been married. Individuals all completed a structured clinical interview administered by a trained interviewer and lifetime diagnoses for alcohol, nicotine, cannabis, cocaine, and opiate dependence were made in a consensus case conference according to Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) criteria.

DNA was derived from whole blood or saliva and extracted and stored at the Rutgers University Cell and DNA Repository following stringent quality control and assurance procedures (www.rucdr.org). Samples were genotyped on 657,366 markers (including 95,876 intensity-only markers) using the Illumina Human 660W-Quad array (Illumina, Inc., San Diego CA) following standard protocol. Details concerning the quality control procedures used with both markers and samples is given in [51]. Briefly, samples were eliminated because of: 1) low call rate; 2) low GenCall score; 3) extreme heterozygosity or homozygosity; and 4) sample mix-ups. Markers were eliminated because the marker had: 1) been identified as problematic by Illumina; 2) more than one mismatch in duplicated samples; 3) a call rate < 99%; 4) a minor allele frequency < 1%; 5) more than two Mendelian inconsistencies; 6) deviation from Hardy-Weinberg equilibrium at $p < 10^{-7}$; and 7) an association with participant sex or batch at $p < 10^{-7}$. European ancestry was based on self-report, confirmed through an EIGENSTRAT analysis [29] as detailed in [51].

NICOTINE ADDICTION GENETICS (NAG) PROJECT AND AUSTRALIAN BIG SIBSHIP PROJECTS (NAG-AUS/BIGSIB; NAG-FIN) [52-53]

The study participants for the Nicotine Addiction Genetics Project (NAG) were enrolled at two different sites: the Queensland Institute of Medical Research (QIMR) in Australia and the University of Helsinki (UH) in Finland. Families for both the Australian and Finnish arms of the NAG were identified through smoking index cases by use of previously administered interview and/or questionnaire surveys of the community-based Australian and population-based Finnish registers of twins [52-53]. The Finnish arm of the NAG project (NAG-F IN, see above for detailed description) recruited families from the Finnish Twin Cohort, which consists of all Finnish twin pairs born between 1938 and 1957 [35]. Families chosen for the Australian arm of the NAG study (NAG-Aus) were identified from two cohorts of the Australian Twin Panel, which included spouses of the older of these two cohorts. The ancestry of the Australian samples

is predominantly Anglo-Celtic or northern European (>90%). We also used data obtained from a third Australian Community-based family study, the Australian Big Sibship (BigSib). The BigSib sample comprises families ascertained through the Australian Twin Panel selected for five or more offspring sharing both biological parents. Families for the BigSib sample were recruited from the same Australian Twin Panel sources as were the NAG Australian families, and phenotypic information was obtained using the same assessment protocol as for the NAG. Clinical data for both Australian and Finnish subjects were collected using a computer-assisted telephone diagnostic interview (CATI), and adaptation of the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) [25, 37] for telephone administration. The tobacco section of the CATI was derived from the Composite International Diagnostic Interview (CIDI) [54] and incorporated standard FTND, DSM-III-R, and DSM-IV assessments of nicotine dependence. It also included a detailed history of cigarette and other tobacco use, including quantity and frequency of use for current, most recent, and heaviest period of use. The measure examined for the purposes of this study was the number of cigarettes smoked per day, during heaviest period of use. All data-collection procedures were approved by institutional review boards at Washington University (WU), the QIMR, and the Ethics committee of the Hospital District of Helsinki and Uusimaa, including the use of appropriate and approved informed-consent procedures. For this meta-analysis, NAG/BigSib-Aus combined sample contributed information from a total of 1329 unrelated adult subjects (about 40% women; including 45% from the BigSib sample), 18-82 years of age (mean age: 44 years) at the time of assessment; including 592 who reported smoking 10 or fewer cigarettes per day, 489 subject who reported smoking 20 to 39 per day, and 248 Australians who reported smoking 40 or more cigarettes per day during their heaviest period of smoking. Participants gave informed consent for an interview, for providing a blood sample for DNA extraction and cell lines, and for the sharing of their anonymous clinical and genotypic records with scientists outside of the NAG and/or BigSib research teams of investigators.

NICOTINE ADDICTION GENETICS - FINLAND STUDY (FTC/NAG-FIN) [52]

The FTC/NAG-FIN sample was ascertained from the Finnish Twin Cohort study consisting of adult twins born between 1938 and 1957 (www.twinstudy.helsinki.fi). Based on earlier health questionnaires, the twin pairs concordant for ever smoking were identified and recruited along with their family members (mainly siblings) for the Nicotine Addiction Genetics Finland study (N = 2,265), as part of the consortium including Finland, Australia, and United States (see NAG-AUS below) [52]. Data collection took place between 2001 and 2005.

The NAG-FIN study provided information on nicotine and alcohol dependence, based on an adaptation of the Semi-Structured Assessment for the Genetics of Alcoholism (SSAGA) [25, 37] for telephone administration. DSM-IV lifetime diagnoses for alcohol and nicotine were assessed using SSAGA. In addition to DSM-IV diagnoses, nicotine dependences from Fagerström Test for Nicotine Dependence (FTND) and Cigarettes Per Day (CPD) measures were assessed. CPD was based on quantity and frequency of use during the heaviest period of use. Lifetime exposure for nicotine and alcohol was also reported in SSAGA. Use and dependence on cannabis and

illicit drugs were asked, but the prevalence was low. All data-collection procedures were approved by the Ethics committee of the Hospital District of Helsinki and Uusimaa and the IRB at Washington University, St. Louis, MO, including the use of appropriate and approved informed-consent procedures.

In the NAG-FIN study, a total of 1,387 Finnish adult twins were genotyped for rs1799971. For this meta-analysis, one twin from pairs in which both co-twins were genotyped was randomly selected for the analyses. This resulted in a sample of 879 unrelated twins (30.0% females) whose mean age at assessment was 54.9 years (sd=4.5 yrs, range: 42.0–76.6 yrs).

DNA was derived from blood samples taken at local health centres and shipped overnight to the National Institute for Health and Welfare. DNA was extracted by standard methods and genotyping was performed at the Welcome Trust Sanger Institute (Hinxton, UK) on the Human670-QuadCustom Illumina BeadChip (Illumina, Inc., San Diego, CA, USA), with quality control methods as previously described [36].

NATIONAL YOUTH SURVEY – FAMILY STUDY (NYSFS; ORIGINALLY "NATIONAL YOUTH SURVEY") (NYS) [55-57]

The National Youth Survey began in 1977. At that time 1,725 adolescents between the ages of 11 and 17 years old as well as one of their parents were interviewed. Participants were chosen by a scientific method designed to select individuals who were representative of the national population. It was a sample of households with all children between 11 and 17 within a chosen household recruited. It is a longitudinal study, with 12 waves of interviews conducted. DNA for 1,072 individuals was collected as part of wave 10 interviews [55-57]. Subjects without genotypic information (N = 82) or phenotypic data (N = 16) were removed from the analysis. Of the remaining 974 individuals, 770 were European American and 120 were African American. Because the original ascertainment methods included all children in the household, we used SAS proc surveyselect to identify the subset of unrelated individuals used in this study (European American N = 552, African American N = 77). All research protocols and consent forms were approved by the institutional review board of the University of Colorado. Phenotypic data used in this study were from the wave 10 data collection, when the initial probands were 37-43 years old. Subjects were assessed with the Composite International Diagnostic Interview-Substance Abuse Module (CIDI-SAM). Cigarettes per day was defined as the number of cigarettes smoked per day at the time when the individual smoked the most in his/her lifetime. Alcohol, cocaine, nicotine, opioid, and cannabis DSM-IV dependence were derived from the CIDI-SAM.DNA was derived from buccal cells. A TaqMan assay performed on a ABI PRISM© 7900 (Applied Biosystems, Foster City, CA) was used to determine the genotype. For QC, genotypes were called independently by two individuals. Samples that were unable to be genotyped (assumed poor quality DNA) or for which two independent callers disagreed were removed from the analysis.

OREGON YOUTH SUBSTANCE USE PROJECT (OYSUP) [58]

The Oregon Youth Substance Use Project is a fifteen-year ongoing longitudinal study of approximately 1000 participants examining the etiology of substance use in Oregon youth [58]. OYSUP began in the 1997-1998 school year with students in five grade cohorts in the first

through fifth grade, recruited from a single school district in a working class community in Western Oregon. Using a stratified random sample, parents of 2,127 students in 15 elementary schools were sent a letter followed by a phone call describing the project and soliciting participation. We obtained parental consent for 1075 students (50.7%) to participate in assessments for the first four years of the study. An average of 215 students per grade (1st through 5th) participated in the study in the first year with an even distribution by gender (50.3% female, N=538). Participants were comparable to elementary students in the district on race/ethnicity and participation in the free-lunch program [58]. However, they had significantly higher scores (albeit a small absolute difference) on academic achievement tests in both reading and math. Students in the study were comparable to students in Oregon on 30 day prevalence of use of all substances in the 6th grade (DHS, State of Oregon, 2000), with the exception of inhalants, where the prevalence of inhalant use was slightly higher in Oregon than in the OYSUP sample.

Lifetime smoking status was defined by an affirmative to the question, "Have you ever smoked at least 100 cigarettes in your lifetime?". Current smoking status was defined by an indication of at least one cigarette in answer to the question, "During the past seven days, how many cigarettes did you smoke on a typical day?". Alcohol abuse or dependence diagnoses were based on the structured clinical interview for DSM-IV Axis 1 disorders, the SCID-I [59]. A lifetime diagnosis was defined as the presence of abuse and/or dependence at any time during the participant's life, and a current diagnosis was defined based on abuse and/or dependence on alcohol present during the last six months.

The study cohort of 404 individuals were aged 21 – 23 within two grade-based cohorts from the OYSUP. Appropriate institutional review board approval was obtained from the Oregon Research Institute and SRI International. We collected saliva for analysis of salivary DNA from OYSUP participants in the afternoon at least two hours after the participant had eaten lunch as described [60]. Saliva biospecimens for DNA extraction were collected using commercially available kits for saliva collection using the manufacturer's protocol. DNA was extracted for N=404 participants using the DNA Genotek protocol modified as described [61]. Samples were genotyped using TaqMan® SNP Genotyping assay (Applied Biosystems) C__8950074_1_ for OPRM1 rs1799971 on a ViiA™ 7 System (Applied Biosystems) with positive and negative controls and 18% duplicates. Genotyping gave 99.8% completion rate with a minor allele frequency of 0.125 and was in Hardy-Weinberg Equilibrium. Regenotyped samples gave 100% concordance rate with original genotypes.

MUNICH GERMANY (PAGES) [62]

Individuals with schizophrenia were ascertained from the Munich area in Germany. Of this sample, 71% were of German descent and 29% were Caucasian middle Europeans. Case participants had a DSM-IV and ICD-10 diagnosis of schizophrenia with the following subtypes: paranoid 78.2%, disorganized 16.7%, catatonic 0.5% and undifferentiated 4.6%. Detailed

medical and psychiatric histories were collected, including a clinical interview using the SCID, to evaluate lifetime Axis I and II diagnoses. Four physicians and one psychologist rated the SCID interviews, and all measurements were double-rated by a senior researcher. Exclusion criteria included a history of head injury or neurological diseases. All case participants were outpatients or stable inpatients. Further details can be found in previous reports [62].

The Structured Clinical Interview for DSM-IV (SCID I and SCID II) [59, 63] was used to obtain Statistical Manual of Mental Disorder IV (DSM-IV) lifetime diagnoses for alcohol, cannabis, cocaine, and opiate. Nicotine dependences from Fagerström Test for Nicotine Dependence (FTND) and Cigarettes Per Day (CPD) measures were assessed as well.

For this meta-analysis, the German sample contributed unrelated European Caucasians (414 schizophrenia patients). The study obtained informed consent from participants and approval from the appropriate institutional review boards.

DNA was obtained from peripheral blood. DNA concentration was adjusted using the PicoGreen quantitation reagent (Invitrogen, Karlsruhe, Germany), and 1 ng was genotyped using the iPLEX assay on the MassARRAY MALDI-TOF mass spectrometer (SEQUENOM, Hamburg, Germany). Genotyping call rates in cases and controls were all >97%. Allele frequencies were similar to CEU sample frequencies. A subsample of SNPs and DNA was genotyped twice to check for genotyping errors.

PATCH IN PRACTICE (PiP) [64]

The Patch in Practice cohort is drawn from participants in a randomised controlled trial of two levels of behavioural support offered in conjunction with nicotine transdermal patch in Primary Care, conducted in 2002-2005, which recruited n = 935 heavy smokers (≥ 10 cigarettes per day). Participants were given sufficient patches for 8 weeks. Abstinence from smoking at 1-week, 2-week and 4-week follow-up was confirmed by expired carbon monoxide concentration (≤ 10 ppm), and at 12-week, 26-week and 1-year follow-up by salivary cotinine concentration (≤ 15 ng/ml). Data collected at baseline included age in years, sex, body mass index in kg/m^2, self-reported ancestry and self-reported smoking habits, including a measure of nicotine dependence using the Fagerström Test for Nicotine Dependence. All participants provided a blood sample collected by the participant's GP or practice nurse. Ethics approval was obtained from the appropriate NHS Research Ethics Committee, and informed consent procedures were followed. Genotyping was conducted as previously described [64].

SMOKING IN FAMILIES (SMOFAM) [65-69]

The Smoking in Families study is a longitudinal, repeated measures age-sequential cohort study of environmental and psychosocial risk factors for adolescent and young adult substance use, including tobacco use and dependence, initiated in 1984 [65]. Subjects were recruited through advertisements in traditional media, and flyers distributed at middle and high schools in four mid-sized and small urban and rural Pacific Northwest cities with populations ranging from 30,000 to 175,000. The original SMOFAM study recruited 763 families, with at least one adolescent age 11 or older. Families with smoking parents and/or adolescents were of special interest since the focus was on adolescents at risk for tobacco and other substance use. Within

each family one adolescent was designated as the proband if s/he had previously tried a substance. Each proband had to have at least one parent agree to participate. An attempt was made to encourage both parents and all sibs over the age of 11 to participate. The only other requirement was that all participants needed to be able to read basic level English. Repeated annual assessment of probands facilitated characterization of longitudinal phenotypes for tobacco use, including the acquisition and maintenance of smoking, as well as many potential psychosocial and environmental predictors of substance. Probands and two first-degree relatives from 158 pedigrees with at least three ever-smoking individuals per pedigree were recruited from SMOFAM to study the environmental, genetic and metabolic determinants of tobacco use, completed a detailed smoking history questionnaire including cigarettes per day and the Fagerström Test for Nicotine Dependence, and provided a whole blood sample for DNA extraction and analysis [66]. All family members gave written informed consent to search for genes related to the development of nicotine dependence. Institutional review boards at Oregon Research Institute, SRI International and the University of California San Francisco gave approval to conduct the research. DNA was genotyped using a custom GoldenGate assay of 1536 SNPs, including rs1799971, to interrogate cholinergic and dopaminergic candidate genes [67], with quality control measures relating to replicate DNA samples and control DNA samples as described [68], and where genotype errors based on Mendelian inconsistency were evaluated using Pedcheck and Merlin as described [69]. Randomly chosen unrelated individuals with complete genotype data were selected from each pedigree for analysis.

THE UTAH GENETICS OF ADDICTION PROJECT (UTAH) [70]

The Utah Genetics of Addiction Project contributed the Utah cohort from a study of genetic risk markers for nicotine dependence and chronic obstructive pulmonary disease (COPD) [70]. The UT cohort was made up of respondents to community advertising for persons who had smoked more than 100 cigarettes lifetime plus a subset of Lung Health Study (LHS) participants originally recruited in Utah. UT participants were not drawn from a psychiatric treatment population, and no medical or behavioral treatments were offered as part of the study. UT volunteers were not excluded simply because they had a lifetime diagnosis of psychosis or Bipolar Disorder, but they were excluded if their current mental status made it impossible for them to complete the questionnaires or interviews. Pulmonary function testing determined 62% of the UT cohort had COPD. Of UT participants, 43% had not smoked for at least 2 years prior to participation in the study. All UT participants were of European descent, and all had smoked more than 100 cigarettes lifetime. DSM-IV Substance Dependence diagnoses were based on SCID-I/P (2001 Final Version) interviews conducted by a clinical research psychologist. Study procedures were approved by the University of Utah IRB. DNA was isolated from peripheral blood lymphocytes collected in Salt Lake City, UT (UT cohort). SNP genotyping methods were previously described[56] and used the SNPlex assay (Applied Biosystems).

VIRGINIA ADULT TWIN STUDY OF PSYCHIATRIC AND SUBSTANCE USE DISORDER (VA-Twin) [71-73]

The VA-Twins were selected from the Virginia Adult Twin Study of Psychiatric and Substance Use Disorder, which was a population-based epidemiology study. Tobacco smoking and nicotine dependence were assessed by the Fagerström Tolerance Questionnaire (FTQ) and/or Fagerström Test for Nicotine Dependence (FTND) during the time of heaviest lifetime nicotine use. In this

study, only regular smokers (defined as those who used at some point in their lives an average of at least seven cigarettes per week for a minimum of four weeks) were included (N = 2388). One subject from each twin pair was selected, and all subjects were of Caucasian ancestry. The study obtained informed consent from participants and approval from the institutional review board of Virginia Commonwealth University. DNA was extracted from buccal brushes. Genotyping was performed with the TaqMan genotyping method. To ensure the quality of genotyping, negative control samples were included in each plate. Genotypes were scored using a semi-automated protocol [74].

YALE-PENN [75-77]

Yale-Penn study participants were recruited for studies of the genetics of drug (opioid or cocaine) or alcohol dependence. Subjects were recruited at five eastern US sites: Yale University School of Medicine (APT Foundation, New Haven, CT), University of Connecticut Health Center (UConn, Farmington, CT), Medical University of South Carolina (MUSC, Charleston, SC), McLean Hospital (Harvard Medical School; Belmont, MA), and the University of Pennsylvania (Philadelphia, PA). Informed consent procedures were followed for this study. Genetic studies of substance dependence disorders and related traits in a subset of this sample have been published [75-77].

All subjects were interviewed using an electronic version of the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA) [78-79] to derive diagnoses for lifetime substance dependence (alcohol, nicotine, cannabis, cocaine and opioid) and other major psychiatric traits according to DSM-IV criteria. The FTND items were embedded directly in the SSADDA assessment. CPD was derived directly from the FTND question: "When you were smoking regularly, how many cigarettes did you usually smoke in a day?" Subjects were excluded from further study if they had ever received a clinical diagnosis of a major psychiatric illness often associated with psychotic episodes (i.e., schizophrenia, schizoaffective, or bipolar disorder)

A total of 2133 African Americans and 1218 European Americans were genotyped from cell lines, blood and in a small number of cases, saliva. Genotyping was done on the Illumina HumanOmni1-Quad v1.0 microarray containing 988,306 autosomal SNPs. GWAS genotyping was conducted at the Center for Inherited Disease Research (CIDR) and the Yale Center for Genome Analysis. Genotypes were called using GenomeStudio software V2011.1 and genotyping module V1.8.4 (Illumina, San Diego, CA, USA).

A total of 44,644 SNPs on the microarray and 135 individuals with call rates < 98% were excluded, and 62,076 additional SNPs were removed due to minor allele frequencies (MAF) <1%. We identified several instances where identical DNA marker profiles were linked to two different interview forms. When demographic information (sex, date of birth, number of reported children) was consistent across interviews, one sample was randomly removed from analysis. When demographic information was inconsistent across interviews, both samples were removed. Reported sex was verified based on the computed average X chromosome heterozygosity; self-reported males with heterozygosity of more than 20% and self-reported females with heterozygosity less than 20% were excluded unless their true identity could otherwise be reconciled. QC is described in more detail in Gelernter et al [75-77].

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Supplementary Text S2: Power calculation details.

All power calculations were performed using Quanto (Gauderman and Morrisson, http://biostats.usc.edu/software.html).

1. Power for prior GWAS consortia meta-analyses of smoking ([1-3]) to detect *OPRM1*.

Parameters:
Disease allele frequency: 0.15
Odds Ratio: 0.93 (nicotine-specific odds ratio in Table 3; reciprocal is 1/0.93 = 1.075)
Log additive disease model (for analysis of heavy-smoking cases and light-smoking controls)
Ratio of cases to controls: 1:1 (maximizes power at a fixed total sample size)
Population risk: 0.20
2-sided test

Sample sizes of the 3 consortia:
1. TAG [1]: N=38,000 (19,000 case-control pairs).
2. ENGAGE [2]: N=31,000 (15,500 case-ctrl pairs).
3. Ox-GSK [3]: N=16,000 (8,000 case-control pairs).

OPRM1 was tested only in the 3 consortia separately; it was not meta-analyzed across all 3 consortia combined. Meta-analysis across all 3 consortia was performed only for selected top loci in each consortium.

Thus we computed
(1) The power to detect this *OPRM1* effect in at least one of the 3 consortia at genome-wide significance (alpha = 5×10^{-8})
(2) To approximate the power for at least one of the 3 consortia to have selected *OPRM1* for follow-up meta-analysis across all 3 consortia, we relaxed the significance threshold to alpha = 3×10^{-5}. Based on supplementary tables in [1-3], the threshold was in fact more stringent for ENGAGE [2] and Ox-GSK [3], and unclear for TAG [1].
(3) Even though rs1799971 was not tested across the three consortia combined, for comparison we calculated power to detect *OPRM1* in the combined consortia (N=85,000).

Power result 1: Power for genome-wide significance (alpha = 5×10^{-8}) in at least one of the 3 consortia is 4%:

Sample sizes and corresponding power for each individual consortium at alpha = 5×10^{-8}:
1. TAG [1]: N=38,000 (19,000 case-control pairs), corresponds to 2.8% power.
2. ENGAGE [2]: N=31,000 (15,500 case-ctrl pairs) gives 1.2% power
3. Ox-GSK [3]: N=16,000 (8,000 case-control pairs) gives 0.08% power

Then, the power to detect OR=0.93 at GWAS significance in at least one of the 3 consortia is given by the formula
Power = 1-(1-(power to detect in TAG alone))*(1-(power to detect in ENGAGE alone))
*(1-(power to detect in Ox-GSK alone))

= 1 - (1 - 0.028)*(1 - 0.012)*(1 - 0.0008)
= 1-(0.972)(0.988)(0.9992)
= 1-0.95956
= 0.040
= 4.0% power.

Power result 2: Power to detect this effect for follow-up in at least one of the 3 consortia is 40%:

Sample sizes and corresponding power for each individual consortium at alpha = 3×10^{-5}:
1. TAG [1]: N=38,000 (19,000 case-control pairs), corresponding to 26.45% power.
2. ENGAGE [2]: N=31,000 (15,500 case-ctrl pairs), giving 16.5% power
3. Ox-GSK [3]: N=16,000 (8,000 case-control pairs), giving 3.05% power

Then, the power to detect OR=0.93 at 5×10^{-5} in at least one of the 3 consortia is given by the formula
Power = 1-(1-(power to detect in TAG alone))*(1-(power to detect in ENGAGE alone))
*(1-(power to detect in Ox-GSK alone))
= 1 - (1 - 0.2645)*(1 - 0.165)*(1 - 0.0305)
= 1 – (0.7355)(0.835)(0.9695)
= 1-(0.59541115375)
= 0.40459
= 40% power.

Power result 3: Rs1799971 was not tested across all three consortia combined. However, if it had been, power to detect OR = 0.93 at alpha = 5×10^{-8} in a combined analysis of N=85,000 is still only 43%.

2. For comparison: Power of the current targeted study to detect the *OPRM1* association with general substance dependence (N=16,908 subjects) is 79.6%.

Parameters:
Disease allele frequency: 0.15
Odds Ratio: 0.90 (general substance dependence liability odds ratio, Table 3)
Log additive disease model
Ratio of cases to controls: 1:0.8654 (corresponds to 9064 cases, 7844 controls, Table 3)
Population risk: 0.2
2-sided test
N=16,908 total: 9064 cases, 7844 controls

Required multiple-test corrected p-value is alpha = 0.00976

Resulting power is 79.6%.

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The Tobacco and Genetics Consortium (2010) Genome-wide meta-analyses identify multiple loci associated with smoking behavior. Nat Genet 42: 441-447.

Thorgeirsson TE, Gudbjartsson DF, Surakka I, Vink JM, Amin N, et al. (2010) Sequence variants at CHRNB3-CHRNA6 and CYP2A6 affect smoking behavior. Nat Genet 42: 448-453.

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Supplementary Figures: Individual Substance Dependence Analyses

Figure S1. Alcohol Dependence

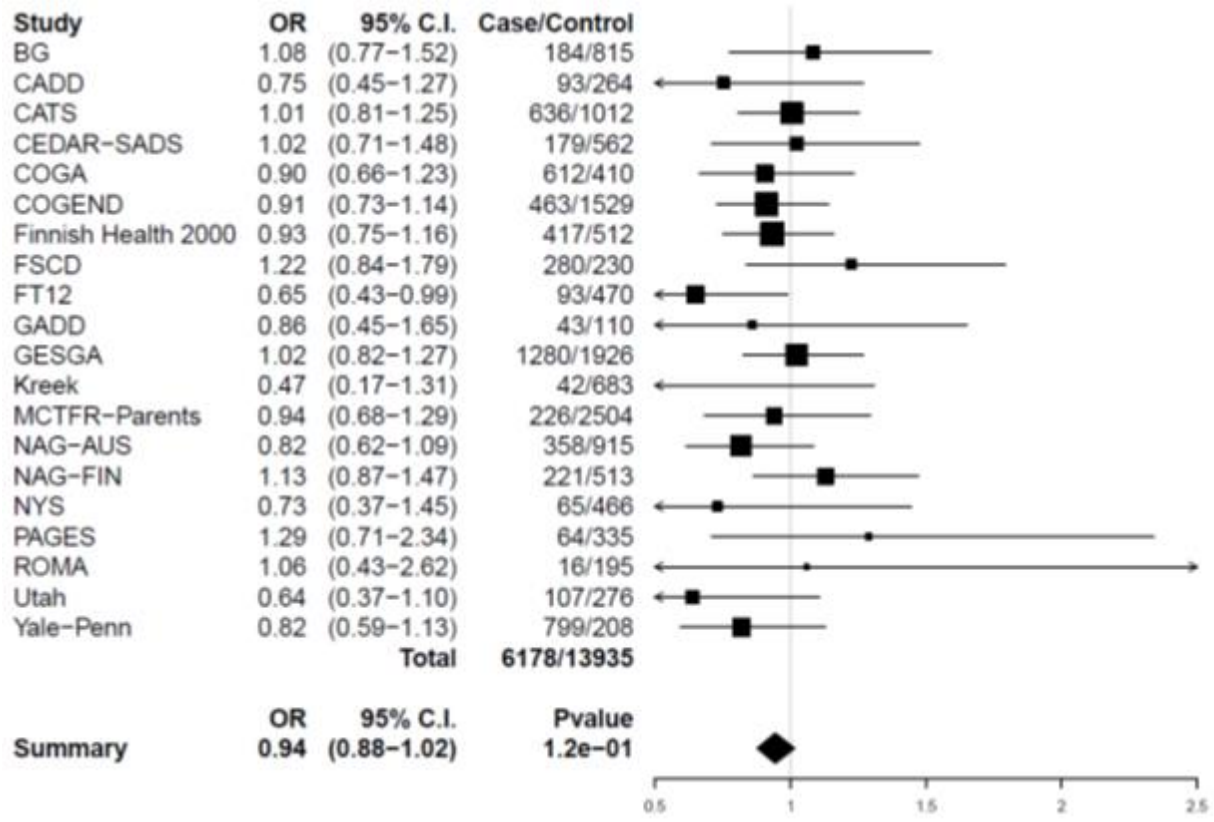

Figure S1 Forest plot of alcohol dependence and rs1799971 across studies that had at least 5 cases (participants with DSM-IV alcohol dependent diagnosis) and 5 alcohol-exposed, non-alcohol-dependent controls (filtered to remove participants who never drank at least one drink in lifetime) each. Summary odds ratio, 95% Confidence Interval and p-values are based on fixed effect meta-analysis.

Figure S2. Cigarettes-per-day (CPD) dichotomized phenotype (heavy vs. light smoking)

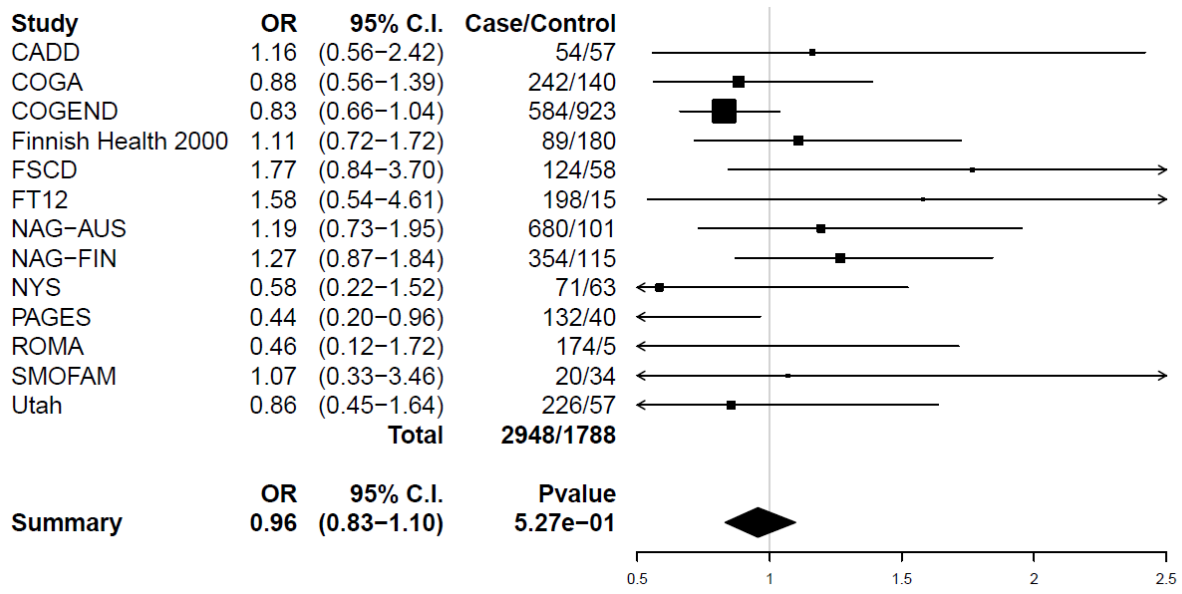

Figure S2 Forest plot of CPD dichotomized phenotype (heavy vs. light smoking) and rs1799971 across studies that had at least 5 cases (participants who smoke more than 20 CPD) and 5 nicotine-exposed controls (participants who smoke less or equal to 10 CPD and have smoked at least 100 cigarettes in lifetime) each. Summary odds ratio, 95% Confidence Interval and p-values are based on fixed effect meta-analysis.

Figure S3. Cannabis Dependence

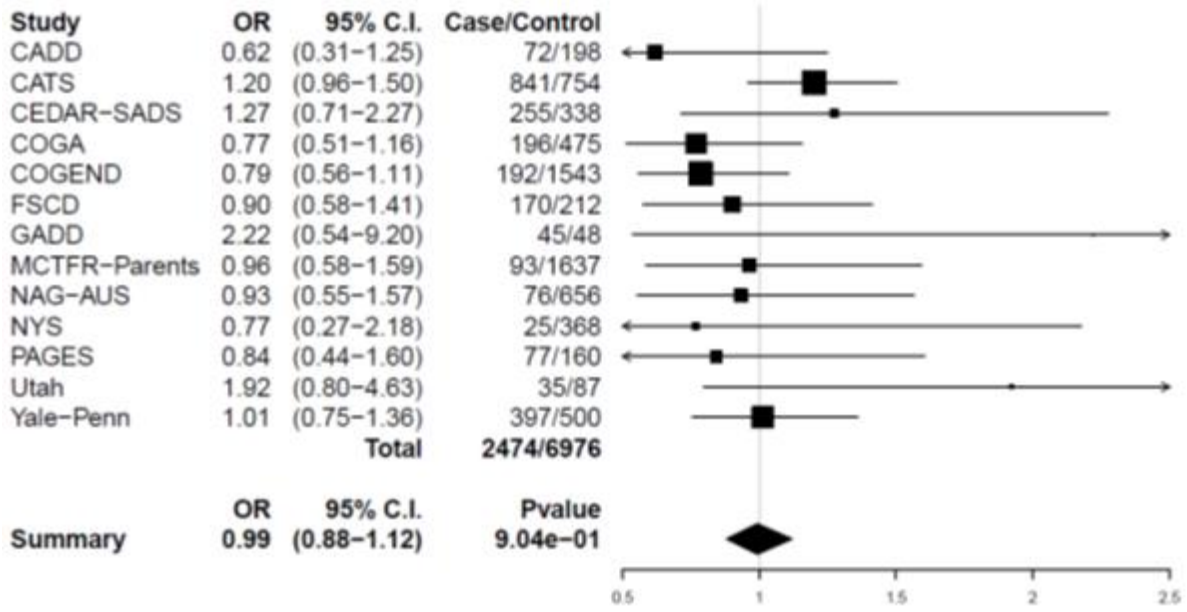

Figure S3 Forest plot of cannabis dependence and rs1799971 across studies that had at least 5 cases (participants with DSM-IV cannabis dependent diagnosis) and 5 cannabis-exposed controls (participants with no cannabis dependent diagnosis and have tried cannabis at least once) each. Summary odds ratio, 95% Confidence Interval and p-values are based on fixed effect meta-analysis

Figure S4. Cocaine Dependence

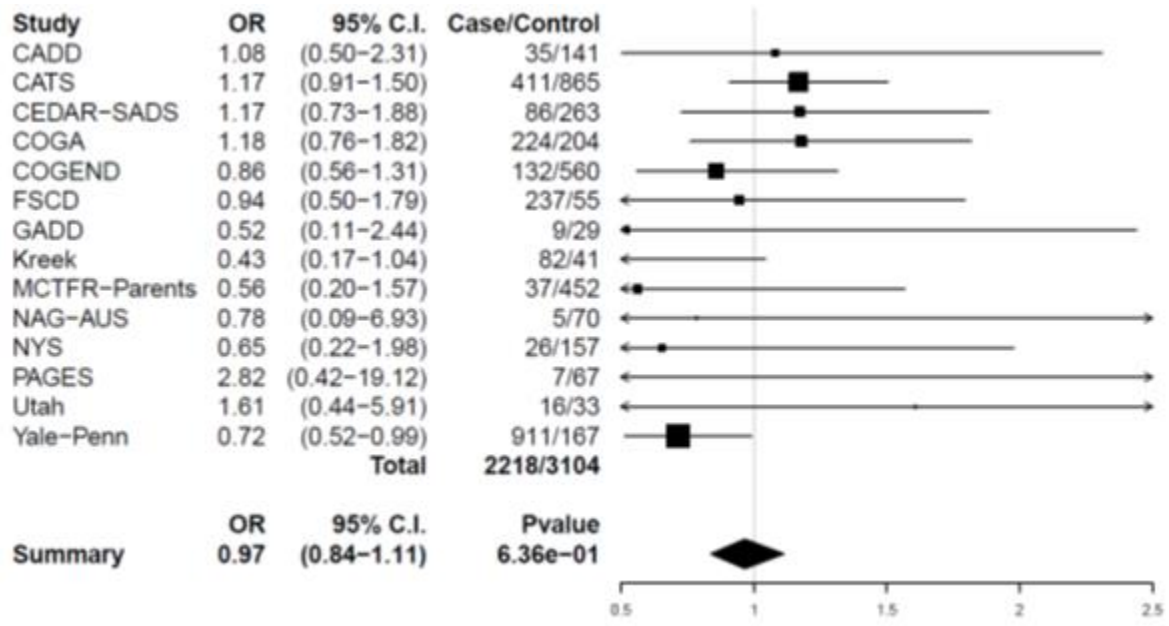

Figure S4 Forest plot of cocaine dependence and rs1799971 across studies that had at least 5 cases (participants with DSM-IV cocaine dependent diagnosis) and 5 cocaine-exposed controls (participants with no cocaine dependent diagnosis and have tried cocaine at least once) each. Summary odds ratio, 95% Confidence Interval and p-values are based on fixed effect meta-analysis

Figure S5. Opioid Dependence

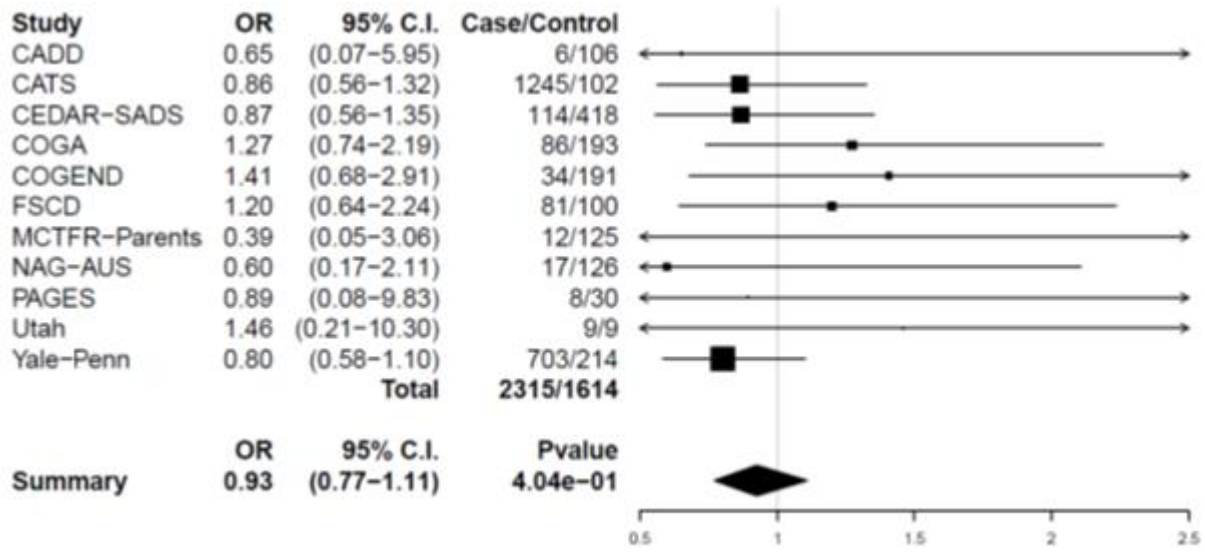

Figure S5 Forest plot of opioid dependence and rs1799971 across studies that had at least 5 cases (participants with DSM-IV opioid dependent diagnosis) and 5 opioid-exposed controls (participants with no opioid dependent diagnosis and have tried an opioid at least once) each. Summary odds ratio, 95% Confidence Interval and p-values are based on fixed effect meta-analysis